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The Examination of Dimethylanthracenes for Carcinogenic Properties*

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The importance of substitution in the 9,10-positions (5, 7) for the development of carcinogenic power in the 1,2-benzanthracene molecule was illustrated when it was found that 9,10-dimethyl-, and 5,9,10-trimethyl-1,2-benzanthracene produced tumors of the skin of the mouse more rapidly; *i.e.*, in 35 days, than did any other compound then known (3). Tests of a large number of benzanthracenes bearing other substituents in these positions have been reported (4, 5, 7). These considerations suggested that substitution in the 9,10-positions might confer carcinogenic power upon a still simpler hydrocarbon, namely anthracene. Accordingly, 9,10-dimethylanthracene, and, for comparison, four other dimethylanthracenes (1,2-, 1,3-, 1,4-, 2,3-), were prepared and tested. Recently Hershberg and Fieser (6) have described compounds, 9,10-dimethyl-1,2-(thiopheno-2′,3′)-anthracene and its selenium isolog, which are related to 9,10-dimethylanthracene.

METHODS

The compounds were administered as follows:

1. By application to the skin of the interscapular region of a solution in benzene (0.3 per cent) twice weekly.

2. By injection *sub cutem* in suspension or solution in glycerol or sesame oil, dose 5 mgm., repeated so that the mice which lived longest had received 12 to 14 doses in from 12 to 22 months. In most cases (Table I) C3H mice were used, as these were found by Andervont (1, 2) to be especially susceptible to induced sarcoma.

3. By insertion of pellets (20 mgm.) *sub cutem*. This method was not very satisfactory as in many cases the pellets sloughed out.

RESULTS

The results are summarized in Table I.

1. The 9,10- compound is the only one which has produced tumors of the skin.

It was applied to two series (A and B) of 20 mice each, and 2 epitheliomas were obtained in each series. In the two series together, 28 out of the original 40 mice were alive at the end of the first year, and of these, 4 mice, which were killed on days 428, 544, 614, and 669, showed epithelioma on the site of application; the last of these animals bore also 3 sebaceous adenomas on the head and neck. The first papilloma appeared in series A on day 490 when 8 mice were alive, and in series B on day 322, when 14 mice were alive.

2. No tumors have been produced by any of these compounds at the sites of injection or insertion into the subcutaneous tissue. This result, in conjunction with 1 above, shows that subcutaneous injection alone is not, when the results are negative, a conclusive test for carcinogenic action. Other instances have been given in publications from this laboratory of compounds which, under the conditions stated, produced epithelioma but not sarcoma: 5-*n*-butyl-, 5-*n*-amyl-, 5-*n*-hexyl-, and 5-*n*-heptyl-1,2-benzanthracene; 1,2,5,6- and 1,2,7,8-dibenzfluorene (4).

3. The lungs were examined carefully with the naked eye, and every portion showing any suggestion of adenoma was excised for microscopic examination; if no such portions were found, or if the sections cut showed no adenoma, the case was recorded as negative. The examination of the whole of both lungs by serial sections would have been impossible in view of the number of animals coming to autopsy. If more than

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one adenoma was found the case was classed as multiple. The great majority of adenomas of the lung in mice lie within fairly well-defined limits of size, and one can draw a useful, though of course arbitrary, distinction between these and others of larger size.

The incidence of adenoma of the lung (Table I) is very suggestive, but such results cannot be regarded as conclusive until the experiments can be carried out upon larger numbers of mice in which the spontaneous incidence of such tumors is known exactly, and such comparisons are made more difficult by differences in in our stock and C3H mice. Until these data are sufficient in number, one can only compare one series of mice with another, as has been done in an earlier paper (4), in which it was recorded that the proportion of lungs showing adenoma was lowest (11 per cent) in mice treated with derivatives of 1,2-benzanthracene, and highest (35 per cent) in the series receiving the 3,4-benzphenanthrenes substituted in the 1- and 2-positions, while intermediate proportions are found in the series receiving acridines (33 per cent), the 6-, 7-, and 8- substituted 3,4-benzphenanthrenes (25 per

TABLE I: RESULTS OF TESTS FOR CARCINOGENICITY OF DIMETHYLANTHRACENES

Dimethyl-anthracene	Method of application	Initial number and strain of mice	Number examined	Number alive after 6 months	Number alive after 1 year	Day of death of last mouse	Epithelioma of skin	Adenoma of lung	Other tumors
1,2-	Painting	10 stock	7	9	7	395	0	0	0
	Injection	10 stock	5	6	3	651	0	0	0
1,3-	Painting	10 stock	8	8	4	417	0	2, days 368, 417	0
	Injection	10 C3H	6	5	2	435	0	0	0
	Pellets	10 stock	3	..	..	557	0	0	0
1,4-	Painting	10 stock	6	8	5	508	0	0	0
	Injection	10 C3H	5	4	1	509	0	0	0
	Pellets	10 stock	7	..	..	495	0	3 adenomas in one, day 417	? Squamous papilloma, stomach.
2,3-	Painting	10 stock	6	8	7	515	0	0	0
	Injection	10 C3H	9	9	7	362	0	1, day 206	0
	Pellets	10 stock	5	..	..	474	0	1? day 212	0
Total		110	67	..	36	...	0	4, one multiple	
9,10-	Painting	40 stock	26	..	28	669	4	8, multiple in 5, large in 3	Carcinoma of kidney; ? malignant growth of pylorus; squamous papilloma, stomach.
	Injection	10 C3H *	9	6	3	442	0	0	2, Mammary carcinoma.
	Pellets	10 stock	7	..	4	533	0	4, multiple and large in one	Squamous papilloma, stomach.
Total		60	42	..	35	...	4	12, multiple in 6, large in 4	

* Another series, of 10 stock mice, of which 5 lived for more than 450 days, received injections in sesame oil; no local tumors were produced. Other tumors were not sought for and hence this series is not included in the Table, but it provides further evidence of the absence of sarcoma production.

length of life in the various series. However, these results, taken as they stand, show that:

a. Of 42 mice receiving 9,10-dimethylanthracene, 12 (29 per cent) showed adenomas, which were multiple in 4 cases, and 6 of the tumors were large.

b. Of 67 mice receiving the 4 other dimethylanthracenes, 4 (6 per cent) showed adenomas, which were multiple in one case, and none were large. The rather shorter life of these mice would tend to produce a difference in the direction observed, but this factor does not seem sufficient to account for the whole of the difference.

Data are being accumulated upon the incidence of spontaneous tumors of the lung, and of other organs, cent), 1,2-dimethylchrysene (26 per cent), and the fluorenes (30 per cent).

4. The only other undoubtedly malignant tumor observed, apart from mammary tumors in C3H mice, was a carcinoma of the kidney in a mouse bearing also an epithelioma of the skin induced by 9,10-dimethylanthracene.

SUMMARY AND CONCLUSIONS

Five dimethylanthracenes (1,2-, 1,3-, 1,4-, 2,3-, 9,10-) were tested for carcinogenic activity by application to the skin, and by subcutaneous injection and implantation, in mice. The 9,10- compound alone produced epithelioma of the skin, and adenomas of the lung

were most abundant in the mice receiving this compound. None of the 5 compounds produced tumors of the subcutaneous tissue. The results indicate: 1. the importance of substitution in the 9,10- positions as a source of carcinogenic activity, and 2. the inadequacy of subcutaneous injection as a sole test for carcinogenic power.

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ADDENDUM

The following was received from Professor Kennaway too late for inclusion in the galley proof:

Bradbury, Bachmann, and Lewisohn injected a single dose (2 mgm. in sesame oil) of 2,9,10-trimethylanthracene *sub cutem* in 15 mice, and obtained no tumors in 8 months.

See: Bradbury, J. T., W. E. Bachmann, and M. G. Lewisohn. The Production of Cancer by Some New Chemical Compounds. Cancer Research, **1**:685-694. 1941.

The Metabolism of Dimethylaminoazobenzene (Butter Yellow) in Rats*

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The purpose of this study was to investigate the metabolism of dimethylaminoazobenzene (butter yellow) in rats.

In 1935, Sasaki and Yoshida (30) reported the production of liver hepatoma by means of *o*-aminoazotoluene in rats ingesting a rice diet which was deficient in some essential constituent. In 1937, Kinosita (20) obtained hepatoma of the liver by feeding dimethylaminoazobenzene to rats ingesting an inadequate diet.

Okada (29), Kinosita (21), Ando (3), and Nakahara and co-workers (27) later reported in similar experiments that the production of liver cancer can be prevented by the addition of rice bran, yeast, or liver extract to the basal diet. The production and prevention of liver tumors as described by these workers has been repeated and extended by Rhoads and co-workers (18, 19, 34).

No experimental data have been reported in regard to the metabolic fate of dimethylaminoazobenzene, although Kinosita (20, 22) suggested that the compound was excreted as aniline and N,N′-dimethyl-*p*-phenylenediamine.

MATERIALS AND METHODS

Adult rats of the Sherman strain employed in this study ingested ad libitum a diet of brown rice. To each 100-cc. portion of the rice, 0.18 gm. of N,N′-dimethyl-*p*-aminoazobenzene (Eastman No. 338), dissolved in 6 cc. of olive oil, was added. The diet was supplemented with 10 cc. of milk per rat per day. Each animal was injected once a week subcutaneously with 40 mgm. of N,N′-dimethyl-*p*-aminoazobenzene dissolved in 1.5 cc. of olive oil. The rats were kept in metabolism cages. The urine was collected daily and stored in the refrigerator. Twelve liters of urine were collected and worked up in 2-liter portions.

The ether-soluble metabolites of dimethylaminoazobenzene excreted in the urine were separated into fractions containing phenolic, neutral, basic, and phenolic-basic compounds. The separation procedure employed is summarized in Fig. 1. Half of the urine was extracted and fractionated in the presence of sodium hydrosulfite to prevent oxidation. The metabolites were isolated from the subfractions and identified after further purification by high vacuum distillation and chromatographic adsorption analysis. A diazotization reaction for the presence of amino groups was used to control the procedures.

A. Color reaction for the amino group.—A color test to detect semiquantitatively the presence of amino groups was used throughout the purification and isolation procedures. The reaction consisted of a diazotization of the amines and the formation of a colored complex. The test is based upon the method of Bratton, Marshall *et al.* (5) for sulfonamides. The material to be tested was dissolved in a measured quantity of methyl alcohol (1 to 25 cc.). Two aliquots (0.2 to 0.4 cc.) were transferred to test tubes. After the addition of 0.5 cc. of 10 per cent hydrochloric acid to each sample, one aliquot was hydrolyzed for 3 minutes over an open flame. The diazotization was then carried out on the hydrolyzed and unhydrolyzed portions. Because of the insolubility of the colored complexes in water, it was necessary to bring the alcohol concentration to 80 per cent before the addition of the coupling reagent.

B. Fractional ether extractions. I. Preparation of ether extracts from urine (AE, BE, CE, DE).—The fractions from urine were prepared by continuous ether extractions for 24 hours in a Kutcher-Steudel apparatus. Four primary ether extracts were obtained from the same urine treated successively as follows: 1. The urine was acidified to pH 1 with concentrated hydrochloric acid (ether extract AE). 2. The urine was then made alkaline to pH 8 with solid sodium carbonate (ether extract BE). 3. The urine was reacidified to pH 1 with concentrated hydrochloric acid and refluxed on a hot plate for one-half hour (ether extract CE). 4. The hydrolyzed urine was made alkaline with solid sodium carbonate to pH 8 (ether extract DE). Each ether extract was evaporated to approximately 150 cc. on a steam bath.

II. Preparation of ethereal fractions containing the phenolic (AE2, CE2) and neutral (AE3, CE3) compounds.—The ether extracts (AE and CE) obtained from acid urine both before and after hydrolysis were treated identically. The ether was extracted with three 30-cc. portions of saturated sodium bicarbonate. The bicarbonate solution containing the acidic compounds

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Scheme for extraction of ether-soluble substances

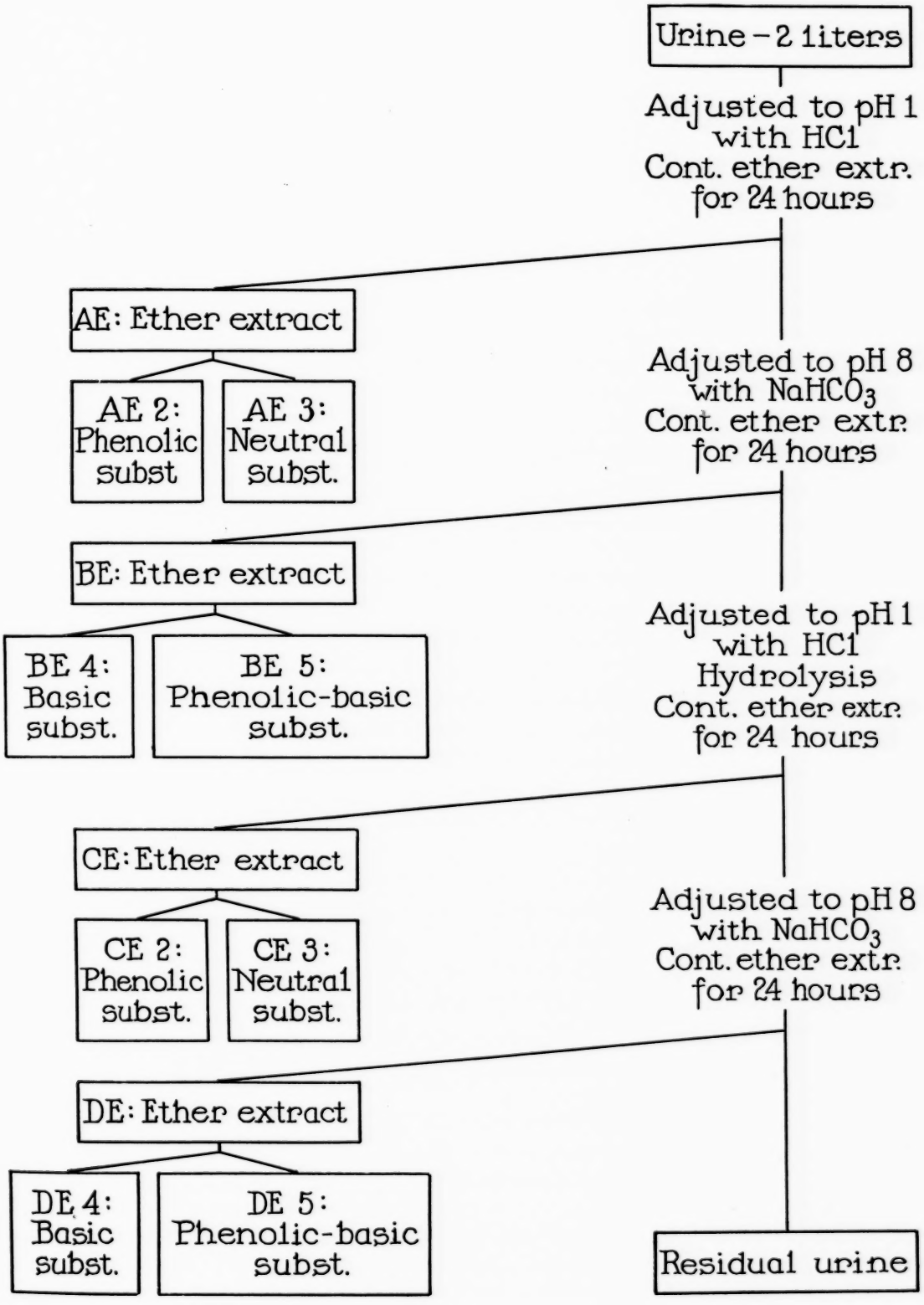
Urine – 2 liters
Adjusted to pH 1 with HCl
Cont. ether extr. for 24 hours
AE: Ether extract
AE 2: Phenolic subst
AE 3: Neutral subst.
Adjusted to pH 8 with $NaHCO_3$
Cont. ether extr. for 24 hours
BE: Ether extract
BE 4: Basic subst.
BE 5: Phenolic-basic subst.
Adjusted to pH 1 with HCl
Hydrolysis
Cont. ether extr. for 24 hours
CE: Ether extract
CE 2: Phenolic subst.
CE 3: Neutral subst.
Adjusted to pH 8 with $NaHCO_3$
Cont. ether extr. for 24 hours
DE: Ether extract
DE 4: Basic subst.
DE 5: Phenolic-basic subst.
Residual urine

FIG. 1

was made acid, hydrolyzed, and gave a negative test for the presence of amino groups; this fraction was discarded. The remaining ether was extracted with three 30-cc. portions of 2N sodium hydroxide. The combined sodium hydroxide solutions were made strongly acid with hydrochloric acid and extracted with three 300-cc. portions of ether. This fraction contained the phenolic compounds (AE2 and CE2). The ether remaining after extraction with sodium hydroxide contained the neutral compounds (AE3 and CE3). The ether extracts were evaporated to dryness under reduced pressure.

III. Preparation of the ethereal fractions containing the basic (BE4, DE4) and phenolic-basic (BE5, DE5) compounds.—The ether extracts (BE and DE) obtained from alkaline urine before and after hydrolysis were treated identically. The ether was extracted with three 30-cc. portions of 10 per cent hydrochloric acid. The residual ether was evaporated; the residue gave a negative test for the presence of an amino group and was discarded. The hydrochloric acid solution containing the basic and phenolic-basic compounds was made alkaline to pH 8 with solid sodium carbonate and extracted with three 300-cc. portions of ether. After evaporation to approximately 150 cc. the ether was extracted with three 30-cc. portions of 2N sodium hydroxide. The ether remaining contained the basic substances (BE4 and DE4). The sodium hydroxide solution was acidified with hydrochloric acid, made alkaline to pH 8 with solid sodium carbonate, and extracted with three 300-cc. portions of ether. This fraction contained the phenolic-basic substances (BE5 and DE5). The ether extracts were evaporated to dryness.

C. Acetylation.—The more stable acetyl derivatives were prepared from amino compounds present in the phenolic-basic (BE5 and DE5) and basic (BE4 and DE4) fractions. The material was dissolved in approximately 300 cc. of ether. According to the quantity of amines present, from 1 to 3 cc. of acetic anhydride were added. The solution was allowed to stand for one-half hour and was then evaporated *in vacuo* over a steam bath. The material was taken up in benzene several times and evaporated to dryness until the odor of acetic anhydride could no longer be detected. The procedure was found satisfactory for monoacetylation of the amino group (16).

D. High vacuum distillation.—High vacuum distillation was carried out with a Hickman molecular still at a pressure below 10^{-4} mm. of mercury. Distillates were collected between the following temperatures: 25-75° C.; 75-150° C.; 150-225° C. High vacuum distillation was employed for crude fractions and large amounts of impurities remained in the residue.

E. Chromatographic adsorption analysis.—Fractions were further purified by fluid chromatographic adsorption analysis. Glass columns between 4.6 and 6.0 mm. in inside diameter were employed. The adsorbent was aluminum oxide Brockmann (Merck). The ratio of aluminum oxide to the crude material was roughly 100 to 1. The column was prepared with the solvent in which the unpurified fraction was dissolved, and the sequence of solvents was continued from that point as described below. Air pressure to force the solvents through the column was supplied by water flowing from a height of 5 feet into a closed container. A calcium chloride tube inserted between the pressure system and the column absorbed the water vapor. The following solvents in one to four 50-cc. portions were used successively: petroleum ether, 50 per cent petroleum ether-ether, ether, 50 per cent ether-acetone, acetone, methyl alcohol, ethyl alcohol. From 10 to 25 eluates resulted from one chromatographic adsorption analysis. The eluates were evaporated to dryness *in vacuo* over a steam bath, dissolved in methyl alcohol, and an aliquot was tested for the presence of amino groups. The strongly positive eluates were combined and the chromatogram was repeated until a satisfactory preparation was obtained. From the eluates of two fractions (BE4 and BE5) several compounds could be separated from each other by the chromatographic technic. To illustrate the chromatographic procedures, the chromatogram of the fraction containing the phenolic bases (BE5) is included in Table I.

RESULTS

Isolation of the Metabolites of Dimethylaminoazobenzene

I. Isolation of N-acetyl-p-aminophenol.—a. N-acetyl-*p*-aminophenol was isolated from the fraction containing phenolic substances (AE2). Crystalline material contaminated with a red oil was obtained from the 50 per cent acetone-ether eluate of the chromatogram. The crude compound was recrystallized twice from acetone-ether mixtures, decolorized with a small quantity of charcoal, and sublimed on the block at a pressure of 10^{-3} mm. of mercury and at a temperature of 130° C.

Analysis: $C_8H_9NO_2$

	N	C	H
Found	9.24	63.73	6.05
Theoretical	9.27	63.57	5.96

m.p.: 169-170° C. (uncorr.); mixed m.p.: 168-170° C. (uncorr.).

b. In the fraction containing the phenolic-bases (BE5), *p*-aminophenol was present. The material in this fraction was acetylated. The procedure of isolation and purification of N-acetyl-*p*-aminophenol from

this fraction was identical with that described under Ia.

Analysis: $C_8H_9NO_2$

	N	C	H
Found	9.34	63.66	6.07
Theoretical	9.27	63.57	5.96

m.p.: 169-170° C. (uncorr.); mixed m.p.: 169-170° C. (uncorr.).

c. In the fraction containing the phenolic bases extracted from hydrolyzed urine (DE5), free *p*-aminophenol was present. The material in this fraction was acetylated and N-acetyl-*p*-aminophenol was isolated as described under Ia.

Analysis: $C_8H_9NO_2$

	N
Found	9.28
Theoretical	9.27

m.p.: 168-169° C. (uncorr.); mixed m.p.: 168-170° C. (uncorr.).

adsorption analysis. The chromatogram was started in ether. N,N'-diacetyl-*p*-phenylenediamine was eluted in 50 per cent acetone-ether. The product was decolorized with charcoal and sublimed on the block at 10^{-3} mm. of mercury at a temperature of 175° C. At higher temperatures decomposition occurred.

Analysis: $C_{10}H_{12}N_2O_2$

	N	C	H
Found	14.46	62.35	6.45
Theoretical	14.58	62.50	6.25

m.p.: sublimes 300-310° C. (uncorr.); mixed m.p.: sublimes 305-310° C. (uncorr.) (rearrangement c. 265° C.).

b. *p*-Phenylenediamine was present in the basic fraction extracted from unhydrolyzed urine (BE4). The material in this fraction was acetylated and the

TABLE I: CHROMATOGRAPHIC ANALYSIS OF PHENOLIC-BASIC FRACTION BEFORE HYDROLYSIS; EXTRACTION RUN WITH SODIUM HYDROSULFITE

Solvent	Amount in cc.	Substance recovered on evaporation of solvent	Color test
50% Petroleum ether-ethyl ether	50	Yellow crystals (needles); m. p. 116°-117° C.; sulfur resulting from use of $NaHSO_2$	—
50% Petroleum ether-ethyl ether	50	Traces of sulfur	—
Ethyl ether	50	Trace of oil	—
Ethyl ether	50	Rhomboidal crystals contaminated with yellow oil; m. p. 154°-156° C.*; unknown compound	+++
Ethyl ether	50	Negative	—
50% Ether-acetone	50	Red pigment	—
50% Ether-acetone	50	Clump crystals coming out of reddish oil on addition of ether; m. p. 169°-170° C.†; N-acetyl-*p*-aminophenol	++++
50% Ether-acetone	50	Traces of N-acetyl-*p*-aminophenol contaminated with pigment and oil	+
Acetone	50	Traces of N-acetyl-*p*-aminophenol; brown pigment	—
Methyl alcohol	50	Dark brown pigment	—
Ethyl alcohol	50	Traces of light brown pigment	—

* Recrystallized from petroleum ether-ether mixtures.
† Recrystallized from 10% acetone-ether mixtures.

II. Isolation of N,N'-diacetyl-p-phenylenediamine.—a. The diacetate of *p*-phenylenediamine was isolated from the fraction containing neutral substances (AE3). Since this fraction contains a large amount of oily impurities, a preliminary separation was necessary. The fraction was washed with three 50-cc. portions of petroleum ether to remove a portion of the oil. The petroleum ether-insoluble residue was extracted with several portions of ether at the boiling point. An equal volume of petroleum ether was added to the combined ether extracts. After standing for several hours, the petroleum ether-ether mixture was filtered. The filtrate and the petroleum ether-soluble fraction were combined and worked up as described under VI. The precipitate from the petroleum ether-ether solution and the ether-insoluble material were combined for further purification by fractional high vacuum distillation. The fraction which distilled between 150-225° C. gave a strong positive test for the amino group and was subjected twice to chromatographic

diacetyl derivative was isolated by chromatographic adsorption analysis, as described under IIa.

Analysis: $C_{10}H_{12}N_2O_2$

	H	C	H
Found	14.57	62.44	6.30
Theoretical	14.58	62.50	6.25

m.p.: sublimes 305-310° C. (uncorr.); mixed m.p.: sublimes 305-310° C. (uncorr.) (rearrangement c. 265° C.).

c. In the fraction from hydrolyzed urine containing the bases (DE5), free *p*-phenylenediamine was isolated. The material in the fraction was acetylated. The purification and isolation procedures are described under IIa.

Analysis: $C_{10}H_{12}N_2O_2$

	N
Found	14.49
Theoretical	14.58

m.p.: sublimes 305-310° C. (uncorr.); mixed m.p.: sublimes 305-310° C. (uncorr.) (rearrangement c. 265° C.).

III. Isolation of an unidentified metabolite.—From the fraction containing the phenolic bases extracted from the urine before hydrolysis (BE5) after acetylation, N-acetyl-*p*-aminophenol was isolated as described under Ia. In addition, a small amount of crystalline material was present in the ether eluate of the chromatogram. After decolorization with charcoal and recrystallization from mixtures of petroleum ether-ether, the compound melted at 156-157° C. (uncorr.). The material crystallized in white, glistening plates. The compound gave a transient color with the amino group color test. After several hours the color redeveloped. Sublimation of this compound was not possible since it is unstable to heat. Analysis was carried out on 2 preparations.

Analysis:

		N	C	H
Found	a.	7.43	60.61	5.83
	b.	7.56		

m.p. 156-157° C. (uncorr.).

IV. Isolation of N,N'-dimethyl-p-phenylenediamine.—From the fraction containing basic substances from unhydrolyzed urine (BE4), after acetylation, a small amount of N-acetyl-dimethyl-*p*-phenylenediamine was eluted in the chromatographic adsorption analysis by ether. This compound was only present in the basic fractions from urines which were extracted in the presence of sodium hydrosulfite: m.p. 131-132° C. (uncorr.); mixed m.p. 131-133° C. (uncorr.).

V. Isolation of aniline.—The basic fraction (BE4) from unhydrolyzed urine prepared in the presence of sodium hydrosulfite was examined after acetylation for the presence of acetanilide. The compound was eluted from the chromatogram in 50 per cent petroleum ether-ether contaminated with sulfur [1] formed during the extraction procedure from sodium hydrosulfite. The eluate was dissolved in a small volume of acetone in which sulfur is practically insoluble. The solution was filtered. The material in the filtrate was then subjected to high vacuum distillation. The fraction distilling between 75-150° C. was taken for chromatographic adsorption analysis. A small amount of crystalline material was obtained from the 50 per cent petroleum ether-ether eluate and recrystallized from petroleum ether: m.p. 114-116° C. (uncorr.); mixed m.p. 114-115° C. (uncorr.).

VI. Attempted isolation of N,N'-dimethyl-p-aminoazobenzene.—The petroleum ether-soluble fraction from the extract containing the neutral substances, described under IIa, was subjected to chromatographic adsorption analysis.[2] No crystalline material was obtained from repeated application of the chromatographic technic. Each fraction was tested for the presence of N,N'-dimethyl-*p*-aminoazobenzene by the addition of 10 per cent hydrochloric acid. In the presence of N,N'-dimethyl-*p*-aminoazobenzene, a red color develops (7). The color test is not specific for N,N'-dimethyl-*p*-aminoazobenzene. Several of the eluates from the chromatograph gave a red color with hydrochloric acid, indicating that minute amounts of N,N'-dimethyl-*p*-aminoazobenzene may be present.

[1] The presence of sulfur inhibits color formation in the amino group test as applied here.

[2] Only those fractions prepared in the absence of sodium hydrosulfite were employed.

DISCUSSION

To prevent oxidation of the unstable amines which result from the metabolism of dimethylaminoazobenzene, sodium hydrosulfite was employed during the extraction and fractionation procedures. Parallel extractions without the use of the reducing agent were also carried out. From both types of extractions the following metabolites were isolated: *p*-aminophenol, N-acetyl-*p*-aminophenol, *p*-phenylenediamine, N,N'-diacetyl-*p*-phenylenediamine, and an unidentified metabolite. Traces of dimethylaminoazobenzene were present in the fractions prepared without the addition of sodium hydrosulfite. From the fractions prepared in the presence of sodium hydrosulfite, small amounts of aniline and dimethyl-*p*-phenylenediamine were isolated. Since sodium hydrosulfite causes reductive splitting of the azo linkage, it is reasonable to assume that these two amines are artificially produced from dimethylaminoazobenzene present in the urine. The yield of the split products of dimethylaminoazobenzene from 12 liters of urine was too small for elementary analysis. Nakamura *et al.* (28) reported the excretion of very small amounts of dimethylaminoazobenzene after subcutaneous injection of this compound.

The scheme in Fig. 2 represents the metabolism of dimethylaminoazobenzene in rats. The azo linkage of dimethylaminoazobenzene is split during metabolism. Cleavage of the azo linkage in metabolism was reported for chrysoidine (33), Orange I (33), and neoprontosil (6). After the administration of *o*-aminoazotoluene, N,N'-diacetyl-*o*-toluenediamine was isolated from the urine of rabbits (8), and after the ingestion of prontosil, sulfanilamide has been recovered from human urine (6).

The aniline portion of dimethylaminoazobenzene is hydroxylated in the para position with the formation of *p*-aminophenol which is partially acetylated in the course of metabolism. The isolation of additional *p*-aminophenol after hydrolysis of the urine indicates that this compound is excreted as conjugated nonether-soluble derivatives. Probably these compounds are the ethereal sulfate and the glucuronide, since several investigators have reported that aniline is converted to *p*-aminophenol which is excreted as the glucuronide

and as the ethereal sulfate in rabbits (32), dogs (23), and man (26). The possibility of the presence of the glucuronide of N-acetyl-*p*-aminophenol must be considered. The conversion of aniline to N-acetyl-*p*-aminophenol has not been reported. However, after the administration of acetanilide, N-acetyl-*p*-aminophenol has been isolated from human urine (25).

place in the course of metabolism. Compounds such as dimethyl-*o*-toluidine (9, 11), dimethylbromtoluidine (11), dimethyl-*m*-aminophenol (11), benzbetain (9), dimethylaniline (10, 13, 14), and methionine (4) have been shown to be demethylated in the animal organism. Compounds such as pyridine (12), nicotinic acid (1), glycocyamine (4, 15), xanthine bases (31),

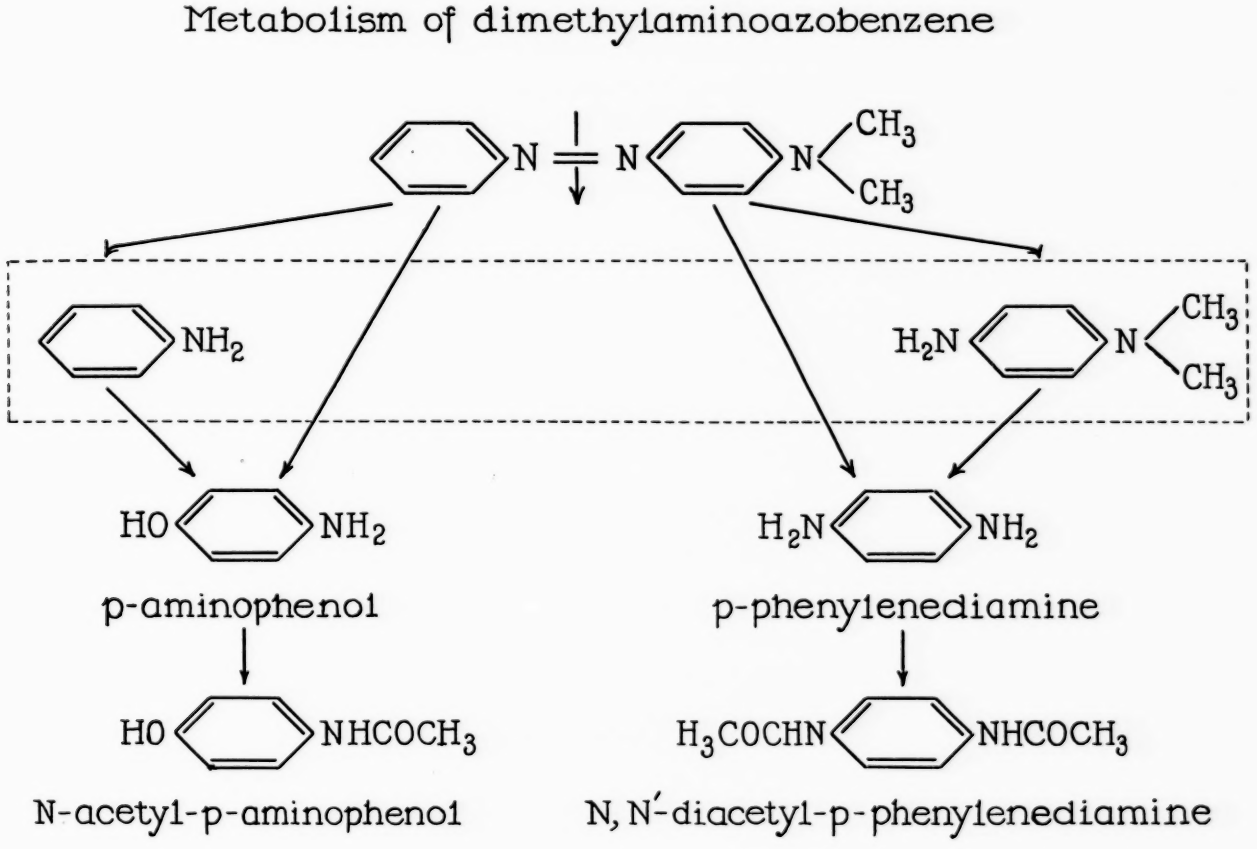

FIG. 2

Another metabolite, probably a derivative of the aniline portion of the dimethylaminoazobenzene molecule, was isolated in very small amounts. The nature of the compound must be established after larger amounts are available.

The dimethyl-*p*-phenylenediamine portion of dimethylaminoazobenzene undergoes demethylation to yield *p*-phenylenediamine, which is excreted in the free and acetylated forms. The free compound has also been isolated after hydrolysis of the urine, but its presence may be accounted for on the basis of the relative insolubility of the acetate in the primary ether extract.

Demethylation and methylation are known to take homocysteine (35), and other amino acids (2) are methylated in the course of metabolism.

The experiments described have offered no conclusion as to the relative speed of reaction of the various metabolic changes which take place in the organism. Experiments are in progress to determine whether the azo linkage is broken before or after the demethylation of the dimethyl-*p*-phenylenediamine portion of the molecule. In addition, it will be of interest to ascertain whether the methyl groups derived from the dimethyl-*p*-phenylenediamine portion of the molecule can function biologically to furnish methyl groups required in intermediary metabolism.

The five basic metabolites of dimethylaminoazo-

benzene isolated in this study have not been tested for carcinogenic activity, but compounds closely related to dimethylaminoazobenzene; namely, dimethyl-*p*-phenylenediamine (20, 22), aniline (20, 22), and *p*-aminoazobenzene (30) have been tested, and do not produce cancer of the liver. Since these compounds were administered orally, they may be oxidized or reduced in the intestinal tract, and thus deprived of a possible carcinogenic activity. Experiments are in progress to test the carcinogenicity of the compounds isolated in this study by injection.

The biological acetylation of *p*-aminophenol and *p*-phenylenediamine is of interest in view of the work of Kensler and Rhoads (17) in which it was shown that acetylation of *p*-phenylenediamine and dimethyl-*p*-phenylenediamine removes the toxic effect of the free compounds to the coenzyme I system *in vitro*. On the other hand, it is known that some acetyl derivatives, for example those of the sulfonamide series, are in some instances more toxic *in vivo* than the free compound (24).

How far the metabolic products isolated or possible intermediary products formed in the course of metabolism of dimethylaminoazobenzene may account for the formation of liver hepatomas remains to be clarified. A study of the fate of dimethylaminoazobenzene under varying dietary régimes may be helpful in elucidating this problem.

SUMMARY

The metabolism of N,N′-dimethylaminoazobenzene (butter yellow) was investigated in rats. The following compounds have been isolated and identified: *p*-aminophenol, N-acetyl-*p*-aminophenol, *p*-phenylenediamine, and N,N′-diacetyl-*p*-phenylenediamine. An additional metabolite, as yet unidentified, has been isolated. The significance of the splitting of the azo linkage and the demethylation of N,N′-dimethylaminoazobenzene in relation to the carcinogenic activity of this compound and its metabolites is discussed.

Elementary analyses were made by the courtesy of Dr. A. Elek, Rockefeller Institute for Medical Research.

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The Effect of Carcinogens on the Hepatic Vitamin A Stores of Mice and Rats

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This study of vitamin A depletion occurring with experimentally induced visceral carcinogenesis was undertaken because it was not feasible to estimate accurately the vitamin A content of epidermis in the systematic study of epidermal methylcholanthrene carcinogenesis in mice, which is being carried out in the Barnard Hospital.

Goerner (8) was the first to show that repeated intraperitoneal injections of 1,2,5,6-dibenzanthracene markedly reduced the vitamin A content and increased the total lipid of hepatic mitochondria of rabbits. Enlargement of the liver and loss of body weight accompanied these changes. Goerner and Goerner (9) also demonstrated that the mitochondria of a transplanted rat carcinoma and sarcoma were lacking in vitamin A, although the hepatic stores of the host were normal. Further experiments of these workers (10) showed that 2-amino-5-azotoluene caused a lowering of the hepatic vitamin A when fed to rats. Cells of liver tumors induced by this azo compound were devoid of vitamin A, but surrounding normal hepatic cells still contained the vitamin. While dibenzanthracene injected intraperitoneally did not affect the liver stores of vitamins C or D, the inhibition of growth in young rats was paralleled by a decrease in hepatic vitamin A (11).

Baumann, Foster, and Lavik (1) placed rats with moderate stores of vitamin A on diets low in this factor and injected separate groups of the animals with colloidal suspensions of 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, benzanthracene, and methylcholanthrene. All of these hydrocarbons caused partial depletion of vitamin A from the liver, but dibenzanthracene proved much more effective than the others. These investigators found no correlation between the carcinogenicity of the compounds and the effect upon vitamin A. For example, butter yellow, a carcinogenic compound, and carbon black, a non-carcinogenic substance, were equally without effect upon the vitamin store. Livers of rats bearing methylcholanthrene-induced subcutaneous tumors contained more vitamin A than non-tumorous rats treated with dibenzanthracene.

Since in these earlier studies attention was concentrated on the depletive action of cancer-producing hydrocarbons on hepatic vitamin A content, without reference to their carcinogenic action, it seemed important to us to include the latter effect in this correlation. Methylcholanthrene was therefore injected intraperitoneally at regular intervals over a long period of time to determine whether a relationship exists between the decrease in hepatic vitamin A and the appearance of tumors in the liver and other viscera of mice.

MATERIALS AND METHODS

All vitamin A determinations were made with an improved microphotoelectric colorimeter which will be described in detail elsewhere (3). Briefly, the density of the 620 mμ. band of the Carr-Price reaction was measured as follows: Red light of *constant* intensity was allowed to pass through the blue solution and then to a recording photoelectric cell for exactly 1½ minutes. The current generated by the latter was stored in a condenser and measured by deflection of a ballistic galvanometer. Fig. 1 shows the calibration curve when a vitamin A concentrate [1] of known potency was used

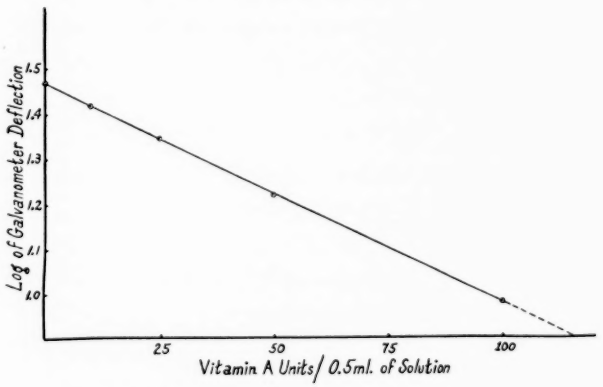

FIG. 1.—Calibration curve for vitamin A.

for reference. The logarithms of the galvanometer deflections are plotted against the U.S.P. units of vitamin A. The units of vitamin A in an unknown could be easily calculated by determining the logarithm of the galvanometer deflection, after which the U.S.P. units of vitamin A could be read directly from the calibration curve.

The entire liver was excised from the animal immediately after killing by a sharp blow on the head. Excess moisture was removed from it by blotting with filter paper, and the tissue then weighed to the nearest

[1] The author is indebted to Dr. Samuel M. Gordon, Endo Products Inc., 395 Fourth Avenue, New York, and to the American Research Products, Minneapolis, Minn., for the supply of the distilled vitamin A concentrate.

milligram. Saponification and extraction with purified ether was carried out according to the procedure of Davies (5). The unsaponifiable fraction was dissolved in chloroform (reagent quality) and diluted to exactly 5 ml. for determination of the vitamin A. Usually 0.5 ml. of this solution sufficed for analysis. To this was added an equal volume of a saturated solution of antimony trichloride in chloroform to develop the color.

The chloroform extracts of the unsaponifiable fractions of mouse livers practically always gave clear colorless solutions. Occasionally, when a liver had an excessive amount of vitamin A, the chloroform solution was tinged with yellow. In such cases the unsaponifiable fraction had to be diluted to 20 to 25 ml. to give 10 to 90 U.S.P. units of vitamin A in 0.5 ml. of solution. Spectroscopically, the Carr-Price reaction for the unsaponifiable fractions of mouse livers showed only one visible absorption band, that at 620 to 625 mμ.

Exploratory experiments revealed that the intraperitoneal injection of methylcholanthrene resulted in ascites, the severity depending upon the amount of this carcinogen administered. These preliminary experiments indicated that in order to reduce mortality from ascites the total amount of this carcinogen administered in small doses for a long period of time should not exceed one mgm. As a convenient concentration, methylcholanthrene (Edcan Laboratories) was dissolved in the fraction of lard melting at or below 37° C. (2) so that 0.05 ml. contained 0.025 mgm. of the hydrocarbon. As a control, the noncarcinogenic hydrocarbon, phenanthrene (Eastman), was used at the same concentration.

Swiss mice, 3 to 4 months old of both sexes, were employed. One hundred and thirty-two mice were divided into 4 groups as follows: 42 for methylcholanthrene (MC); 30 for phenanthrene (PH); 30 for lard controls (L); and 30 for untreated controls (C). Each mouse of the MC group received 0.025 mgm. methylcholanthrene twice weekly; each of the PH group, 0.025 mgm. phenanthrene twice weekly; each of the lard controls, 0.05 cc. of this substance alone twice weekly. Injections were usually made 4 days apart. When malignant tumors appeared, portions were removed for histological study.

Mice were fed Rockland Mouse Pellets and water ad libitum.

RESULTS WITH MICE, GROUP I

The results are shown in Fig. 2. For analysis, 4 mice from the methylcholanthrene-treated group and 3 from each of the other groups were killed at the times indicated. Livers were removed and the vitamin A contents determined separately on each liver and averaged to the figures given. At and after 91 days, 3 mice from each group were killed for analysis. The total dosage of the hydrocarbons, at the time the analysis was made, is indicated on the graph. It can be seen that there was no significant change in the vitamin A content per gm. of liver in the 4 groups.

The vitamin A stores of the untreated controls varied from 140 to 295 U.S.P. units of vitamin A per gm. of liver. With one exception, that of the phenanthrene-treated group at 110 days, the vitamin A content per gm. of liver for the 4 groups was between 135 and 320 U.S.P. units of vitamin A per gm. of liver.

The first ascites occurred at 38 days even with the small dose (0.30 mgm.) of methylcholanthrene. The first visceral tumors appeared at 68 days, and at 124 days 10 mice had developed malignant growths. Nevertheless, the vitamin A stores were unchanged. Even the occurrence of extensive ascites, together with tumors that bound the intestinal tract together, did not appreciably affect the content of vitamin A in the livers.

Table I-a shows the occurrence of ascites and of visceral tumors in mice in which analyses were made, and Table I-b gives the same data for mice which died before analysis could be performed. Fortunately, the dose of methylcholanthrene administered was small enough to allow the experiment to extend beyond 100 days. From 108 to 124 days the remaining mice had either tumors or ascites. Not a single case of ascites was observed in the phenanthrene- or lard-treated groups. Nor were there any significant sex differences to the response of the mice to methylcholanthrene. Liver tumors were not observed in any of the methylcholanthrene-treated mice.

Therefore, under the conditions of this experiment, methylcholanthrene did not appreciably affect the vitamin A content of the livers of mice. Tumors were produced in other organs but not in the liver with a minimum carcinogenic dose of around 0.5 mgm. This is in agreement with the work of Shear, Stewart, and Seligman (18) who found that the direct implantation of 1,2,5,6-dibenzanthracene, 3,4-benzpyrene, and 20-methylcholanthrene into the livers of 53 mice resulted in only 4 hepatomas and 1 adenocarcinoma.

Histopathology of Tumors Induced by Methylcholanthrene and Factors Involved in Ascites Production [2]

Thirteen malignant tumors were produced by the intraperitoneal injection of methylcholanthrene. All of these tumors were spindle cell sarcomas, the origin of which was difficult to determine. Most of them were rich in mitotic figures, indicating rapid growth. Invasion of musculature and of adipose tissue gave

[2] The author wishes to express his gratitude to Dr. Zola K. Cooper for histological classification of these tumors.

evidence of malignancy. The tumors also had occasional giant cells and large cells with irregularly shaped nuclei. Tumors appeared in 4 animals in the neighborhood of the kidneys, one of which was invaded. In 3 other mice, there were malignant growths invading respectively the pancreas, the small intestine, and the prostate. Histological examination of the liver did not reveal any abnormal growths, either primary, metastatic, or invasive.

One might note with interest the differences in the production of ascites by the intraperitoneal injection of these hydrocarbons. Methylcholanthrene has a decided tendency to induce ascites even when small amounts are administered over a rather short period of time. Benzpyrene has the same property, but experiments with dibenzanthracene, although limited, indicate that this hydrocarbon has the characteristic to but a slight degree, if at all. Phenanthrene was not

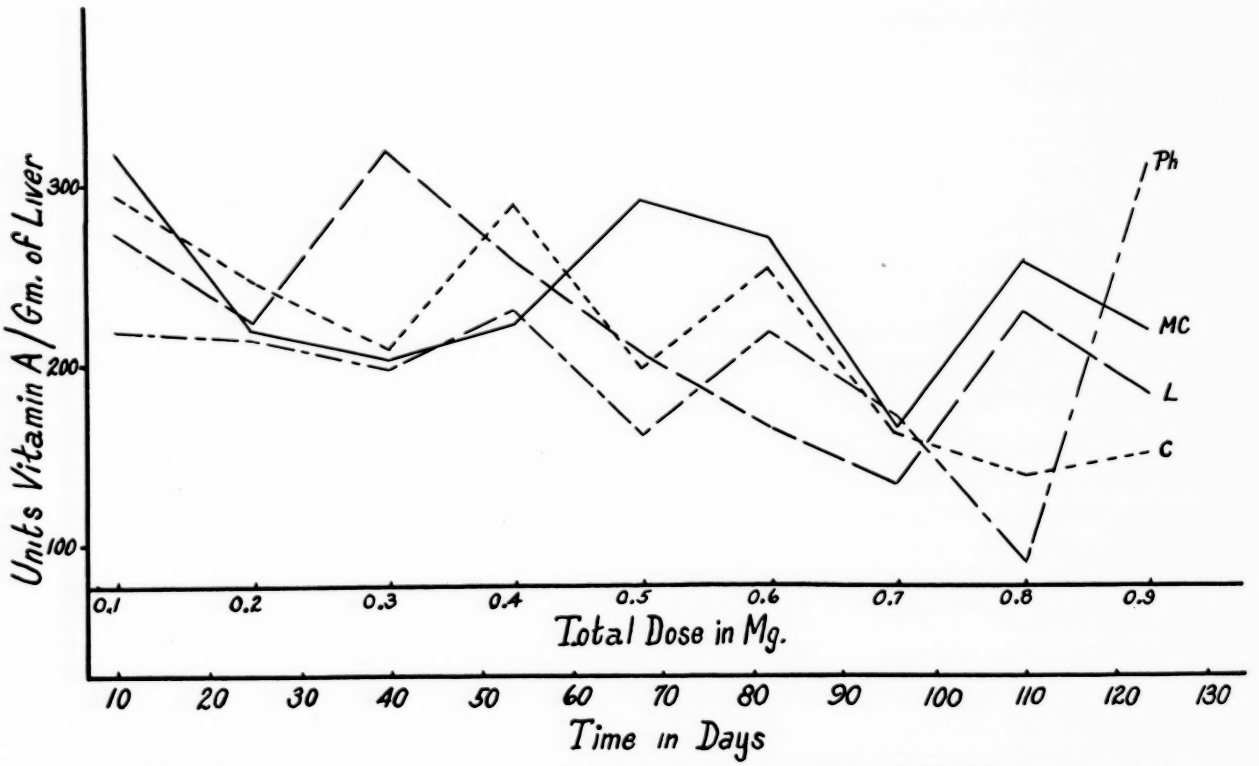

FIG. 2.—The influence of lard solutions of hydrocarbons and of the solvent upon hepatic vitamin A content of mice. PH = phenanthrene; MC = methylcholanthrene; L = lard; C = untreated controls.

TABLE I: OCCURRENCE OF ASCITES AND TUMORS IN MICE INJECTED INTRAPERITONEALLY WITH METHYLCHOLANTHRENE *

Number of mice	Male	Female	Dose of MC, mgm.	Number of tumors	Occurrence of ascites and tumors (days)	Degree of ascites			
						+++	++	+	o
			A. HEPATIC VITAMIN A DETERMINED						
4	4	0	0.3	0	38	0	0	1	3
4	4	0	0.4	0	52	2	0	1	1
3	0	3	0.5	1	66	1	0	0	2
4	4	0	0.6	2	80	1	0	2	1
3	2	1	0.7	2	94	2	0	1	1
3	1	2	0.8	3	108	0	1	1	1
2	1	1	0.85	2	124	1	0	0	1
			B. HEPATIC VITAMIN A NOT DETERMINED						
2	0	2	0.7	2	72- 96	2	0	0	0
3	2	1	0.58-0.65	0	79-107	1	1	0	1
2	2	0	0.5	0	87- 96	0	1	1	0
4	1	3	0.85	1	108-123	0	0	1	3

* Letters and symbols indicate the following: MC = methylcholanthrene; +++ = extensive; ++ = moderate; + = slight; o = none.

found to have induced a single case of ascites. Lorenz and Stewart (15), on the other hand, reported that dibenzanthracene after oral administration could produce ascites in mice and a low grade inflammatory lesion of the small intestines more frequently and more severely than did methylcholanthrene.

In another experiment the carcinogenic action of methylcholanthrene choleic acid, phenanthrene choleic acid, and desoxycholic acid was investigated. The complexes of the hydrocarbons were made according to the method of Fieser and Seligman (7). They were dissolved by triturating 100 mgm. of each with 1 ml. of 0.2 N sodium hydroxide and solution maintained by adding 100 ml. of phosphate buffer of pH 8. One mgm. injections (subcutaneously) were given mice of strain C57 black twice weekly for 14 weeks. At the end of this time one group had received 28 mgm. of methylcholanthrene choleic acid (equivalent to 5.2 mgm. methylcholanthrene); another group had received 29 mgm. phenanthrene choleic acid (equivalent to 3.6 mgm. phenanthrene); and a final group had received 30 mgm. of desoxycholic acid. However, no ascites was observed, and although methylcholanthrene choleic acid induced subcutaneous sarcomas at the site of injection, liver tumors were not found. Phenanthrene choleic acid and desoxycholic acid did not produce any tumors. The lowering of the pH of these choleic acids when injected under the skin of the mice probably caused precipitation of some of the choleic acids. It would seem that the liver has the property of removing cancer-producing hydrocarbons either by detoxification or by metabolizing them so rapidly that an abnormal growth response is not elicited. The results suggest that irritating action of the hydrocarbons when injected intraperitoneally might play a part in the production of ascites.

RESULTS WITH RATS

In attempts to lower the vitamin A stores of young rats, each of 3 groups of 10 animals was given one of the following: methylcholanthrene (MC); phenanthrene (PH); 3,4-benzpyrene (BzP). Doses of 3 mgm. in 0.5 ml. lard were injected intraperitoneally twice weekly for 2 weeks, and then once weekly for one week. On the 4th and final week the remaining rats (which had not yet been sacrificed for analysis) received an additional mgm. of hydrocarbon, or a total of 16 mgm. At 14, 28, 41, and 55 days, 2 rats from each group were killed, the livers removed, and the vitamin A content determined as mentioned above. Ten untreated animals served as controls. Rats of both sexes (96 to 118 gm.) were employed. The diet was Purina Dog Chow with unlimited water. Body weights were recorded every 5 days. The results of this experiment are shown in Table II.

TABLE II: INFLUENCE OF HYDROCARBONS ON THE GROWTH AND HEPATIC VITAMIN A STORAGE IN RATS *

Hydrocarbon	Mgm. injected	Time in days: Of treatment	Time in days: After last injection	U.S.P. units of vitamin A per gm. liver	Per cent hepatic vitamin A †	Per cent of normal growth	Rats with ascites: Males	Rats with ascites: Females
C	0	14	..	68-238 (153)	100	100	..	..
PH	9	14	4	60-178 (119)	78	92	0	0
BzP	9	14	4	64 (64)	52	77	0	1+
MC	9	14	4	13- 89 (51)	33	80	1+	0
C	0	28	..	153-247 (200)	100	100	..	..
PH	16	28	1	136-196 (166)	83	86	0	0
BzP	16	28	1	60-119 (89)	48	72	1+	0
MC	16	28	1	90-102 (96)	45	70	0	1+
C	0	41	..	135-160 (147)	100	100	..	..
PH	16	41	14	87-148 (117)	79	87	0	0
BzP	16	41	14	24- 56 (40)	27	64	1+++	0
MC	16	41	14	0- 16 (8)	5.4	68	1+++	2+
C	0	55	..	106-249 (177)	100	100	..	..
PH	16	55	28	150-213 (182)	102	89	0	0
BzP	16	55	28	123-137 (130)	73	64	0	0
MC	16	55	28	73-111 (92)	46	74	0	0

* Average figures are indicated in parentheses. Letters indicate the following: C=control; PH = phenanthrene; BzP = 3,4-benzpyrene; MC = methylcholanthrene; + = trace; +++ = extensive.
† Indicates per cent of normal where normal is considered 100 per cent.

Because of the wide variation of the hepatic vitamin A content of the normal rat (68 to 249 U.S.P. units per gm. of liver), a depletive effect of the hydrocarbons would have to be extreme to be significant. At 14 and 28 days, after each rat had received 9 and 16 mgm. of each hydrocarbon, respectively, the vitamin A stores of the benzpyrene- and methylcholanthrene-treated groups were nearly half of the normal, while in the phenanthrene-treated group they were about 78 per cent of that of these untreated animals. At 41 days, 14 days after the last injection, the stores of the methylcholanthrene-treated group were almost

exhausted, those of the benzpyrene-treated group being 27 per cent of the controls, while those of the phenanthrene-treated group were still nearly 80 per cent of that of the control rats. At 55 days, 28 days after the last injection, the vitamin A contents were approaching normal, although the methylcholanthrene-treated group was still below 50 per cent. However, the inhibitory effect of methylcholanthrene and benzpyrene on the growth rate persisted.

The unsaponifiable fraction of the rat livers, which was diluted to 20 to 25 ml. for analysis, was always light yellow in color unless vitamin A content was low or absent. However, after 30 seconds (when the determinations were made), no interference due to other carotenoids in the rat livers could be detected by spectroscopic examination of the Carr-Price reaction for vitamin A.

Goerner found that the retardation of growth induced in the rat by 1,2,5,6-dibenzanthracene was paralleled by the decrease in the vitamin A content of the liver. Such was not the case with the hydrocarbons used in this study, although there was some correlation in the phenanthrene-treated group. On the contrary, the rate of depletion of the vitamin A stores in the benzpyrene- and methylcholanthrene-treated groups was greater than the inhibition of growth which was nearly the same for these hydrocarbons. Five rats which received methylcholanthrene and 3 which received benzpyrene developed ascites. Other investigators, Haddow *et al.* (13), Lee (14), and White and White (19) have demonstrated an inhibitory effect of hydrocarbons on the growth of the rat.

RESULTS WITH MICE, GROUP 2

Since it was found that methylcholanthrene and phenanthrene had no appreciable effect upon the vitamin A stores of the mouse (Fig. 2) in the doses given, larger amounts of phenanthrene (PH), 3,4-benzpyrene (BzP), methylcholanthrene (MC), and 1,2,5,6-dibenzanthracene (DiBz) were injected in an attempt to affect the vitamin A stores. Thirty male Swiss mice, 3 to 4 months old, were divided into 5 groups of 6 each. One group served as untreated controls, while each mouse of the other 4 groups received bi-weekly injections of 0.3 mgm. of one of the above-named hydrocarbons dissolved in lard for 7 weeks, and 2 injections of 0.6 mgm. during the 8th week. Each mouse also received 1 drop of Mead's Oleum Percomorphum (containing about 1,400 U.S.P. units of vitamin A per drop) 3 times weekly. It was hoped that by maintaining a large hepatic reservoir of vitamin A, some depletive action of the hydrocarbons could be demonstrated. Vitamin A analysis was carried out as before.

Because of the wide variation in the stores of vitamin A, it is rather difficult to draw any definite conclusions (Table III). At 41 days, when the remaining mice of each group had received a total of 4.5 mgm. of hydrocarbon (1.2 mgm. during the last week), the liver stores of vitamin A were large, but the variation again was rather great. Goerner (8) has already shown that dibenzanthracene in some way prevents the storage of vitamin A in the liver of rabbits, even when accompanied by an excess of carotene or vitamin A administered parenterally. Apparently this does not hold for mice with the hydrocarbons we used and when massive doses of vitamin A are administered orally. Although the mice received large doses of the hydrocarbons, ascites occurred only in the benzpyrene- and methylcholanthrene-treated mice, and this was slight.

TABLE III: INFLUENCE OF LARGE DOSES OF HYDROCARBONS ON THE HEPATIC VITAMIN A CONTENT OF MICE WHEN LARGE DOSES OF VITAMIN A ARE ADMINISTERED ORALLY *

Number of mice	Time of treatment, days	Dose of hydrocarbon, mgm.	Time after last injection, days	U.S.P. units of vitamin A per gm. of liver					Occurrence of ascites	
				C	PH	DiBz	BzP	MC	Number of BzP	Number of MC
2	9	0.9	3	310	203	165	210	505	0	0
2	23	2.1	3	392	221	243	450	293	0	1++; 1+
2	41	4.5	1	992	1704	1392	1841	1066	2+	2+

* Letters indicate the following: C = control; PH = phenanthrene; DiBz = 1,2,5,6-dibenzanthracene; BzP = 3,4-benzpyrene; MC = methylcholanthrene; + = trace; ++ = moderate.

Further investigation will have to be undertaken to explain the differences in response of the mouse and rat to these hydrocarbons with respect to their effect on the hepatic vitamin A stores. A species difference in the metabolism of cancer-producing hydrocarbons, as suggested by the work of Dobriner, Rhoads, and Lavin (6), or a species variation in the chemical configuration of the vitamin A may account for the difference.

In the last experiment the total amount of the hydrocarbons given mice was comparable to that given to the rats on a body weight basis. It has already been noted that the chloroform extract of the unsaponifiable fraction of the liver of mice was invariably colorless unless very rich in vitamin A. On the other hand, colorless solutions from the unsaponifiable fraction of rat livers (which were usually of a light yellow color) meant little or no vitamin A. It does not seem possible that this color difference can be due to wide variation

in vitamin A content since the unsaponifiable fraction of rat livers of average weight of 8 to 10 gm. was diluted to 20 to 25 ml., whereas the same fraction from a mouse liver of average weight 1.5 gm. was diluted to 5 ml. Furthermore the U.S.P. units of vitamin A per gm. of normal mouse liver for 30 normal mice averaged 224 (extremes 140 to 290), while for 10 normal rats the average was 169 (extremes 68 to 249) per gm. of liver. It might also be noted that these hydrocarbons induced not only an increased storage of hepatic vitamin A, but also did not interfere with their assimilation when large doses of vitamin A were administered orally to mice (Table III). The opposite is true for the rat and rabbit according to Baumann (1) and Goerner (8), respectively.

The importance of using more than one species of animals for cancer research is emphasized by an experiment of this type. Whether or not the process of carcinogenesis by hydrocarbons *in situ* differs in various species remains to be investigated.

On the other hand, the work of Claude (4), Polson (16) on rabbits, of Schabad (17) on guinea pigs, and of Goerner and Goerner (12) on rats showed that pronounced liver damage may result from treatment with methylcholanthrene and dibenzanthracene. Apparently this is not the case with mice, since the livers did not show pronounced degenerative changes either in our studies or in those of Lorenz and Stewart (15).

SUMMARY AND CONCLUSIONS

1. Repeated biweekly intraperitoneal injections of mice with lard solutions of methylcholanthrene and phenanthrene for 18 weeks did not affect the hepatic stores of vitamin A. Methylcholanthrene induced rapidly growing sarcomas and the production of ascites. Tumors of the liver were not observed. Neither phenanthrene nor lard produced tumors or ascites.

2. The influence of large doses of intraperitoneally administered phenanthrene, 3,4-benzpyrene, and methylcholanthrene on both hepatic vitamin A storage and growth in young rats was also studied. Methylcholanthrene produced a marked reduction in hepatic vitamin A, and 3,4-benzpyrene had a comparable but somewhat less pronounced effect. Phenanthrene exhibited the least potency, only reducing the hepatic stores about 20 per cent of the normal. With methylcholanthrene and benzpyrene, the depletion of hepatic vitamin A was much greater than the inhibition of growth. In the case of phenanthrene, there was some correlation between the decrease in hepatic vitamin A and retardation of growth. Methylcholanthrene and 3,4-benzpyrene produced ascites in some of the rats.

3. The intraperitoneal administration of large amounts of phenanthrene, 3,4-benzpyrene, methylcholanthrene, and 1,2,5,6-dibenzanthracene in the presence of massive doses of vitamin A administered orally neither appreciably affected the vitamin A stores of the liver of mice nor interfered with the assimilation of the vitamin. Ascites induced by methylcholanthrene was less pronounced in the presence of added vitamin A. Although 3,4-benzpyrene produced ascites in several mice, this reaction did not occur in mice treated with dibenzanthracene or phenanthrene.

4. Possible explanations for the differences in response of the mouse and rat to these hydrocarbons are discussed. The use of more than one species of animal, at least for certain phases of experimental cancer research, would seem to be indicated.

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Choline in Tumor-Bearing Animals and a Choline-Like Effect of Butter Yellow*†

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There are several reasons for examining the relation between choline and tumors. Tumor tissue is relatively high in phospholipid, and malignant tumors contain more than benign tumors (3, 20, 34). The concentration of phospholipid measured as organic P is higher in the healthy growing periphery of rat carcinosarcoma 256 than in the nongrowing necrotic center of the tumor (14). The turnover of phospholipid is higher in tumors than in many normal tissues, although not so high as in liver or intestinal mucosa (4, 15). Tumor lecithin, which contains choline, has a higher rate of turnover than cephalin, which does not (16). Rusch and Kline (30) have observed that carcinogenic hydrocarbons inhibit the autoxidation of phospholipids in the presence of certain natural catalysts. Choline itself is important in normal physiological processes (1, 2, 5, 9) particularly in connection with reactions involving a transfer of methyl groups (6). One of the most potent carcinogens for the rat, butter yellow, contains methyl groups which could theoretically enter into physiological transmethylation reactions. Choline and cystine together have been reported to reduce the incidence of tumors due to butter yellow (13).

Information therefore appeared desirable on the choline content of tumors and of the various tissues of tumor-bearing animals. The growth of preformed tumors and the survival of tumor-bearing mice were determined on diets either high or low in choline, and finally butter yellow was substituted for choline in the prevention of kidney hemorrhages in rats on a choline-free diet.

EXPERIMENTAL

Choline and phospholipids in tumors.—Healthy Flexner-Jobling carcinoma tissue was freed as much as possible from its adherent necrotic material, and approximately 5 gm. was ground with sand and extracted for 3 minutes with 35 cc. of a boiling 1:1 alcohol-ether mixture. The solution was then filtered into a 100 cc. volumetric flask and made up to volume with the alcohol-ether mixture. Two 35 cc. aliquots were taken for the determination of total choline by the reineckate method (17) and two 10 cc. aliquots for the determination of organic P by the method of Fiske and Subbarow (8). The tumor samples analyzed and the results are given in Table I. An average of 1.2 mgm. of choline were present per gm. of fresh Flexner-Jobling periphery, while only 0.7 mgm. of choline per gm. were present in the necrotic center of the same tumor. Since only 0.11 mgm. of choline per gm. of periphery existed in the free form (see below), the difference, 1.2 − 0.11 or 1.09 mgm. per gm., was present as bound choline. This was taken as an index of the amount of lecithin (mol. wt. approx. 778) present, although a small fraction of the bound choline probably existed as sphingomyelin.

The content of organic phosphorus ranged from 0.62 to 0.76 mgm. per gm. of fresh Flexner-Jobling periphery (Table I). This agrees with the results of Uromoto (31) for the same tumor and also with those of Haven (14) for rat carcinosarcoma 256 when expressed in the same units. The organic P values were considered an index of the total phospholipid present. A comparison of the lecithin P, as calculated from the bound choline content of the tissues, with the organic P content as determined directly, revealed that lecithin constituted only about 37 per cent of the total phospholipid in the tumors.

The specificity of the chemical method for choline in tumor tissue was investigated as follows: choline reineckate was precipitated in the usual way, the choline was liberated by treatment with Ag_2SO_4, converted to acetyl choline, and its effect measured on frog muscle under standard conditions (17). The results of the biological determinations averaged 82 per cent of the results by chemical analysis (Table II). While the discrepancy between the two groups of results probably represents the extreme range of variability in the biological method, much better agreement

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between results was obtained when extracts from normal tissues were analyzed by the two methods (17). Hence the possibility exists that choline reineckate from tumor extracts may contain other reineckates as well. If the results of the biological determination of choline should be the more accurate, the percentage of lecithin in tumor phospholipids would be only 30 per cent.

Free and total choline in tissues of tumor-bearing animals.—Free choline was determined as follows: approximately 1 gm. of tissue was immersed in 15 cc. of 10 per cent aqueous CCl_3COOH, and minced with scissors. The tissue was extracted for one-half hour at room temperature with occasional stirring, filtered, and washed twice with 2 to 3 cc. portions of 10 per cent CCl_3COOH. Five cc. of 2 per cent ammonium reineckate in methyl alcohol were then added to the filtrate, and the mixture allowed to stand in the cold for 12 hours. The precipitated choline reineckate was then treated as before (17). A serious potential source of error in this determination was the possible liberation of free choline from choline-containing complexes in the extract. However, pure lecithin (24) was not hydrolyzed in contact with 10 per cent of CCl_3COOH for one-half hour. Furthermore, the amount of free choline determined in either rat or mouse liver, whole or minced, was not altered by exposure to air for one hour. Free choline was extracted as completely from minced tissue as from tissue ground with sand. The tissues analyzed and the results are listed in Table III. No very marked difference in choline content between the tissues of normal and tumorous animals appeared except possibly in the free choline content of the spleen which was decreased in the tumorous animals from a normal of 1.05 mgm. per gm. to an average of 0.69 mgm. per gm. This may have been due to variations in the size of the spleen.

TABLE I: CHOLINE AND ORGANIC P IN TUMOR TISSUE

Tumor	Choline per gm. fresh tissue, μgm.	Lecithin per gm. fresh tissue, mgm.*	Lecithin P per gm. fresh tissue, μgm.*	Organic P per gm. fresh tissue, μgm.	Organic P as lecithin, per cent
Flexner-Jobling					
Sample 1	1,391	7.16	285	758	37.6
Sample 2	1,198	6.06	241	618	39.0
Sample 3	1,245	6.61	263	619	42.5
Rat epithelial-MC †	442	1.84	74	268	27.6
Rat mammary adenofibroma	500	2.17	86	236	36.4
	602	...	...	...	...
Mouse epithelial BP ‡	935	...	...	...	...
		Choline per gm. of necrotic tissue, μgm.			
Flexner-Jobling					
Sample 4	895	662	...	...	...
Sample 5	1,149	718	...	...	...
Sample 6	1,231	...	...	...	...

* Calculated from the contents of "bound choline" in the tissues.
† Primary tumor from the base of the ear due to the local application of methylcholanthrene for 10 months.
‡ Pooled sample of primary skin tumors of 3 mice which had been painted with benzpyrene for 4 months.

TABLE II: COMPARISON OF CHEMICAL AND BIOLOGICAL METHODS FOR THE DETERMINATION OF CHOLINE IN FLEXNER-JOBLING TUMORS

Tumor sample	Chemical method μgm. of choline	Bioassay method μgm. of choline	Per cent recovery
1 Duplicates	2,138	1,760	82.5
	2,220	1,720	77.5
2 Duplicates	2,882	1,990	69.0
	2,882	2,280	79.0
3	1,952	1,915	98.1

TABLE III: FREE AND TOTAL CHOLINE IN CERTAIN TISSUES FROM TUMOR-BEARING ANIMALS

	Rat with Flexner-Jobling carcinoma	Rat with mammary tumor	Mice with benzpyrene tumors	Non-tumorous rats
	mgm./gm. fresh tissue			
Total choline				
Liver	2.3	2.4	2.2 Av.	2.3
Kidney	1.7	2.0	2.4 Av.	2.0
Spleen	1.5	1.4	1.4 Av.	1.6
Brain	2.9	3.0	..	3.0
Free choline				
Liver	0.57-0.77	..	0.67 Av.	0.69
Kidney	0.54-0.61	..	..	0.70
Spleen	0.57-0.80	..	..	1.05
Tumor periphery	0.10-0.12	..	..	..
Tumor center	0.12-0.15	..	..	..

The failure of tumors to alter the choline content of the remainder of the animal was emphasized in the extreme case of a 249 gm. rat bearing a mammary adenofibroma which weighed 146 gm. (total

weight, 395 gm.). The remaining tissues averaged 0.91 mgm. of choline per gm. (Table IV), as compared to 0.92 mgm. per gm. (0.73 to 1.27) for nontumorous rats (17, 18). This parallels the constancy in choline content observed in rats on widely dissimilar diets (17).

On the basis of the comparative lipid content of the periphery and center of rat carcinosarcoma 256, Haven suggested that cholesterol esters and neutral fat are formed from the fatty acids arising from the hydrolytic decomposition of phospholipids (14). This should give rise to free choline. However, although the total choline present in necrotic material was quite low, we have failed to observe any appreciable increase in free choline in the necrotic, nongrowing portion of Flexner-Jobling tumors. This would be contrary to the Haven hypothesis unless the liberated choline were rapidly destroyed. A difference between tumors is also possible; Haven worked with the rat carcinosarcoma 256, whereas our results were obtained with Flexner-Jobling carcinoma.

TABLE IV: THE CHOLINE CONTENT OF TUMOR-BEARING RATS

	Rat 1 mammary tumor	Rat 2 epithelial tumor *
	mgm. choline/organ	
Tumor	73.3	3.1
Kidney	2.9	3.5
Liver	30.3	20.2
Brain	4.7	5.9
Carcass	187.0	228.0
Total	298.2	260.7
Weight of rat (gm.)	395	232
Weight of tumor (gm.)	146	4
Mgm. choline/gm. of (rat + tumor)	0.75	1.12
Mgm. choline/gm. of (rat — tumor)	0.91	1.13
Mgm. choline/gm. tumor	0.50	0.68

* Primary tumor induced by the local application of methylcholanthrene.

The effect of a choline-free diet and of excess choline on tumor growth and on the survival of tumor-bearing animals.—Forty mice bearing epithelial warts caused by methylcholanthrene were divided into two equal groups. One group received a choline-free diet, and the other an additional 0.05 per cent of choline. The choline-free diet consisted of steamed egg white, 18 parts; a mixture of choline-free rice bran concentrate (17) and cooked starch, 24 parts; cerelose, 49 parts; corn oil, 5 parts; Wesson salts (32), 4 parts. Two mgm. of carotene and 1 mgm. of calciferol were added dissolved in the corn oil and 2 mgm. of thiamin and 4 mgm. of riboflavin were added in alcohol solution per kg. of ration. The mixture of cooked starch and choline-free rice bran concentrate was prepared by diluting 300 gm. of the concentrate with 250 cc. of water, mixing it thoroughly with 1,200 gm. of the cooked starch, and drying it in air. The size of the tumors and the survival of the mice were noted at intervals, and the experiment was discontinued at the end of 2 months. On both diets, tumor growth was retarded as compared to growth on a grain ration, and some of the mice appeared to develop a crust on the back in place of the tumor originally present. The mean survival was 38 days both in the presence and absence of choline.

The effect of choline deficiency was also determined on the growth of inoculated tumors. Ten comparable rats newly inoculated with Flexner-Jobling rat carcinoma were divided into two equal groups. One group received the choline-free diet and the other group received the same diet plus 7 mgm. of choline per rat per day. The tumors were palpated once a week and drawn to size, and at the end of 3 weeks they were excised and weighed. The tumors in the two groups were approximately equivalent in size at all times during the experimental period. Tumors from rats receiving the choline-free diet averaged 5.4 gm. in weight (2.8 to 7.6), as compared to 6.6 gm. (3.1 to 8.7) for the tumors from rats receiving choline. Thus dietary choline did not appear to be indispensable for tumor growth. Inasmuch as the rat possesses the ability to synthesize choline (6, 17), the tumor probably satisfies its choline requirement by drawing upon the host. Another possibility is that the tumor itself synthesizes choline.

The effect of excess choline was studied as follows: 2 groups of 20 mice each bearing epithelial tumors due to methylcholanthrene were fed a standard stock diet (19) with or without the addition of 0.3 per cent of choline. No difference between groups was noted in either the size of the tumor, or in the survival of the mice—56 and 57 days mean, respectively. Thus, amounts of choline nontoxic to the mouse were likewise nontoxic to the tumor. It is also probable that the mouse, like the rat, possesses the ability to destroy choline.

The prevention of kidney hemorrhages by butter yellow.—Studies on hemorrhagic kidneys in young rats fed deficient rations have revealed that the condition may be prevented by any of several compounds containing methyl groups; *viz.*, choline, betaine, methionine (9-11). Apparently the rat has a definite requirement for "labile methyl" groups which, if present in adequate amounts, can be converted into essential compounds *in vivo* by transmethylation reactions (12). Butter yellow, N,N-dimethyl-*p*-aminoazobenzene, resembles the compounds which prevent kidney lesions

in that its methyl groups are attached to nitrogen rather than to carbon.

The production of hepatomas by butter yellow is much more sensitive to dietary variations than are the other methods of inducing tumors (25-27). Diets high in protein and in the vitamin B complex (21, 25) markedly reduced the carcinogenicity of the dye, and the suspicion has been expressed that the protective agents may act by destroying the carcinogen rather than by interfering with the fundamental carcinogenic process (25). This destruction might be effected in part by the demethylation of the butter yellow, since *p*-aminoazobenzene, or demethylated butter yellow is not carcinogenic (23). The methyl groups released could then conceivably act like any other "labile methyl" groups. Accordingly an attempt was made to demonstrate an effect of butter yellow on hemorrhagic kidneys, in other words, a labile methyl effect.

TABLE V: THE EFFECT OF BUTTER YELLOW AND OF RIBOFLAVIN ON THE INCIDENCE OF KIDNEY HEMORRHAGES IN RATS ON A CHOLINE-FREE DIET

Group	Butter yellow	Riboflavin per rat daily, μgm.	Rats with kidney lesions *	Average gain in weight, gm.†
1	..	0	4/4	8
2	..	10	6/7	9
3	..	20	3/3	2
4	..	60	4/4	9
5	..	5,000 ‡	3/4	5
6	+	10	0/3	8
7	+	20	1/3	10
8	+	60	0/7	5

* Numerator = number of rats with hemorrhagic kidneys. Denominator = number of rats in group.
† Over the 8-day experimental period.
‡ 1 mgm. riboflavin per gm. of ration.
+ = 1 mgm. butter yellow per gm. of ration.

It might be expected that butter yellow would donate its methyl groups most effectively under dietary conditions which retard tumor production; *i. e.,* high casein plus high riboflavin (21, 25). Unfortunately, however, the severity of kidney hemorrhages is reduced by high levels of casein without the addition of choline or of any other methyl donator (11). Accordingly, we were obliged to feed amounts of protein which were suboptimal for protection against tumors. Variations in riboflavin, however, could be introduced without themselves affecting the kidney lesions (Table V).

Thirty-five rats, 35 to 40 gm. in weight, were divided into 8 groups and fed a choline-free basal ration consisting of extracted casein (29), 18 parts; cerelose, 73 parts; Wesson salts, 4 parts; and corn oil, 5 parts. The daily diet was supplemented with 10 μgm. thiamin, 100 μgm. pantothenic acid, 20 μgm. pyridoxine, 10 μgm. carotene, and 5 μgm. calciferol. In addition, riboflavin and butter yellow were fed as indicated in Table V. The butter yellow was recrystallized from alcohol. After 8 days the rats were killed and the incidence of kidney hemorrhage determined. All rats receiving butter yellow, with one exception, had normal kidneys; whereas, with only one exception, rats which received the basal diet only developed hemorrhagic kidneys (Table V). The lesions have been adequately described elsewhere (7, 9).

The rats receiving butter yellow consumed about 20 to 30 per cent less than the rats on the basal diet. Griffith and Mulford (12) have shown that kidney hemorrhages can be prevented by restricting food intake. Hence the food consumption of rats receiving our basal diet was restricted to that of rats fed butter yellow and furthermore, aminoazotoluene, 4′-amino-2,3′-dimethylazobenzene, which contains no N-methyl groups, was fed for comparison.

Thirty-two rats, 35 to 40 gm. in weight, were divided into four equal groups. Group 1 received the basal diet ad libitum. Group 2 received the basal diet plus 1 mgm. of butter yellow per gm. of ration ad libitum. Group 3 received the basal diet plus 1 mgm. of aminoazotoluene (recrystallized from alcohol) per gm. of ration. Group 4 received the basal ration in amounts restricted to that consumed by group 2. The consumption of each group was measured daily. After 8 days the rats were killed and examined for hemorrhagic kidneys.

Kidney hemorrhage resulted in all rats not receiving butter yellow (Table VI). Restricting the consumption of the basal ration to that on the ration containing butter yellow (ca. 70 per cent of normal intake) did not prevent kidney hemorrhage, and the feeding of aminoazotoluene, which restricted consumption to about 87 per cent of the normal intake, likewise was without effect. It would thus appear that butter yellow prevented kidney hemorrhage by means other than by merely reducing food consumption.

In this series many of the rats consuming butter yellow did not grow, although control animals receiving the same amount of food gained an average of 10 gm. during the 8-day experimental period (Table VI). Thus the dye appeared to restrict growth per se (25, 33). Apparently, however, a failure to grow does not in itself protect the rat against kidney lesions, which develop readily in stunted animals on an arachin diet (7, 18). Rats on aminoazotoluene grew less than the controls, but nevertheless 100 per cent of them developed kidney lesions. On the other hand, many rats in groups 6, 7, and 8 (Table V), which received butter yellow, grew as well as the controls, yet failed to develop kidney lesions. Within groups no correlation existed between the rate of growth of individuals and the degree of kidney damage. Therefore, it ap-

pears that the prevention of kidney lesions by butter yellow is a direct effect, with the inference that butter yellow can act as a methyl donor. Our experiments do not, of course, indicate whether transmethylation occurs before or after the azo linkage is cleaved. Only a very small fraction of the butter yellow ingested is ever recovered from the animal as a pigment (22, 28).

TABLE VI: THE EFFECT OF BUTTER YELLOW AND OF AMINOAZOTOLUENE ON THE INCIDENCE OF KIDNEY HEMORRHAGES IN RATS, COMPARED WITH THE EFFECTS OF REDUCED FOOD INTAKE

Group	Diet	Rats with kidney lesions *	Food intake/ rat/day, gm.	Weight gain/rat, gm.†
1	Low choline	7/8	6.9	15
2	Low choline + butter yellow	0/8	4.9	0
3	Low choline + aminoazotoluene	8/8	6.0	5
4	Low choline, restricted	8/8	4.7	10

* Numerator = number of rats with hemorrhagic kidneys. Denominator = number of rats per group.
† Over the 8-day experimental period.

SUMMARY

Analyses for free choline, total choline, and organic P revealed that less than half of the tumor phospholipids were present as lecithin. The tissues of tumor-bearing animals contained normal amounts of both free and bound choline. A choline deficiency failed to alter the course of development of the two types of tumors studied, and an excess of choline was equally without effect.

The addition of butter yellow to a choline-free ration prevented the development of kidney lesions in young rats. The effect was not due to a reduced food intake, nor could it be duplicated with aminoazotoluene. Thus, like certain other compounds containing labile methyl groups, butter yellow may act as a "methyl donor."

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Absence of Carcinogenicity of a Cod Liver Oil Concentrate*

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The possibility that cod liver oil concentrates might have carcinogenic activity and thus endanger their users was given consideration when a nonsaponifiable lipid extract of human liver injected into mice produced sarcomas (14). The preparation of this carcinogenic human liver extract involved saponification followed by extraction with a lipid solvent, a procedure not unlike that used in the preparation of cod liver oil concentrates [U. S. Patent 1.690.091] (9), some yellow bone marrow extracts [U. S. Patent 2.091.730] (8), some ovarian extracts, and some other biologicals used in medication. Since the possibility that the carcinogenic activity of the human liver extracts might be due to a chemical artifact was not at first excluded (although Schabad (12) prepared a human liver extract which had carcinogenic activity by a method which did not utilize saponification) it was not unreasonable to wonder whether similar artifacts might not be formed in the preparation of cod liver oil concentrates.

Accordingly a nonsaponifiable lipid extract was prepared from cod liver oil by procedures identical with those previously used in the preparation of a human liver extract (16). This was then tested for carcinogenic activity. While this test was in progress other reports appeared showing that the nonsaponifiable lipid fraction of human liver was carcinogenic (4, 6, 7, 11, 15). These results were additional justification for the experiments reported here.

It is also important to examine for carcinogenic activity certain things present in the environment of Americans and North Europeans, and not present among some other peoples, which might explain differences in the incidence of cancer of various portions of the alimentary tract, particularly the stomach (1, 13). In contrast with the low incidence of such cancers in the Bantu of South Africa (2) is their common occurrence in the Afro-American (5, 10). The explanation might lie in differences in the environment, the genetic constitution presumably not having been totally changed.

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PROCEDURE

To 3,100 gm. (3,317 cc.) of a commercial cod liver oil was added 6,200 gm. of 95 per cent ethyl alcohol containing 775 gm. of commercial KOH. This was heated on the steam bath under a reflux condenser for 4 hours, after which it was extracted 3 times with ethylene dichloride. The extract was evaporated to dryness under reduced pressure at 50° C. The residue was resaponified. The nonsaponifiable lipid fraction recovered after repeated extractions with ethylene dichloride was 9.1 gm.

This extract was tested for carcinogenicity by subcutaneous injection in mice. Each mouse received one injection consisting of approximately 250 mgm. of the extract dissolved in about 0.75 cc. of sesame oil. The mixture was heated to 160° C. to aid solution. The mice were 41 to 46 days old at the time of injection. They were of our own strain, previously reported as susceptible to sarcoma induction by crude extracts and hydrocarbons (13, 15). The number of mice of each sex and their survival to several time periods are shown in Table I. The sexes are given separately because their susceptibility to induction of sarcomas has appeared to differ.

TABLE I: TEST FOR CARCINOGENICITY OF A COD LIVER OIL CONCENTRATE

Time in months	Living mice		
	Male	Female	Total
0	15	22	37
6	13	21	34
9	9	20	29
12	4	20	24
15	3	20	23
18	3	16	19
19	2	10	12

RESULTS

No sarcomas have appeared at the site of injection although the experiment was begun with 37 mice, 19 of which are living at the end of 18 months, and 12 at 19 months. Tumors have appeared at distant sites in accordance with the approximate incidence for such

tumors in this colony of mice. The extract has been given a fair test for carcinogenicity as indicated by residues of the original extract at autopsy in many of the animals dying after 1 year.

SUMMARY

A cod liver oil concentrate prepared by saponification and extraction with ethylene dichloride has not been carcinogenic when injected into mice.

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The Nonadditive Effect of Ultraviolet Light and Other Carcinogenic Procedures*†

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Since both ultraviolet rays and the chemical hydrocarbons have been used extensively in the experimental production of cancer the question arises whether a similar mechanism is involved in the formation of tumors by the two types of agent. Certain factors appear common to both. When proper technics are used, each can induce cancer of the skin in about the same length of time. Each involves a latent period, and tumors due to either may only become visible many months after the initial exciting agent has been withdrawn (25). Diets high in fat accelerate the production of tumors due to either agent (2, 3, 16), and the local application of cholesterol in oil likewise accelerates both types of carcinogenesis (14, 24). These similarities may, however, be essentially superficial; the assumption of a similarity in carcinogenic mechanism would certainly be strengthened if it could be shown that one carcinogen could replace the other during the precancerous period (13).

A synergism between two dissimilar carcinogens was demonstrated by Rous and associates (18, 22) when benign warts which had been induced in rabbits' ears by the application of tar became large and invasive after the intravenous injection of Shope papilloma virus. Neither agent by itself induced malignant tumors so quickly. Similarly, Ahlstrom and Andrewes (1) observed an intensified response to fibroma virus when tar was injected intramuscularly. The regression of the lesions was delayed markedly and some fibromas grew to resemble malignant neoplasms. The combined application of ultraviolet irradiation and carcinogenic chemical has not, however, yielded very striking results. Increased cancer formation has been reported due to the combination of ultraviolet light and tar (11, 28), but the observations have not been confirmed with pure hydrocarbons (24, 27), and some workers have actually noted a decrease in the carcinogenic potency of benzpyrene applied to animals kept in the sunlight (10, 20). It is also of interest that benzyprene applied to the skin of a 4-year-old child with xeraderma pigmentosa reduced the sensitivity of the painted area to light (8).

It would appear that an additive effect between two or more carcinogens could be most easily demonstrated when the dose of each alone is subcarcinogenic or capable of inducing only a small percentage of tumors by itself. If the influence of these agents during various stages of carcinogenesis is to be determined, the substances obviously must each be administered during different periods. The purposes of the following experiments, therefore, were to observe the effects of light and hydrocarbon and of light and virus under the conditions outlined, to test the effect of various noncarcinogens on the rate of cancer production with ultraviolet light, and to extend Hieger's observation of an additive effect of hydrocarbons (13).

MATERIAL AND METHODS

Young adult ABC albino mice of both sexes were used throughout. They were kept in groups of 20 to 24 in ordinary metal box cages with water and dog biscuit available at all times. For irradiation the mice were transferred to individual wire holders (25) and exposed to 30,000 ergs/cm^2/sec.[1] from an air-cooled "Uviarc" mercury vapor lamp for 30 minutes daily 6 days a week. The various chemicals tested for modifying effects were crystalline substances obtained commercially. The 9,10-dimethyl-benzanthracene was prepared in this laboratory by Dr. E. A. Prill. Solutions were applied with camel's hair brushes directly to the outer surface of the ear, where irradiation tumors usually develop. All animals were inspected for tumors at biweekly intervals.

The effect of two carcinogenic hydrocarbons applied to the same area during successive periods.— As a basis for the evaluation of possible summation between hydrocarbon and ultraviolet light, it was necessary to determine the extent of the additive effects exerted between the different hydrocarbons

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[1] Determined with the uranyl oxalate actinometer (25).

themselves. Hieger (13) has reported the development of only two tumors in 10 mice treated successively with benzpyrene and dibenzanthracene. Accordingly 9,10-dimethyl-1,2-benzanthracene, 3,4-benzpyrene, and 20-methylcholanthrene were applied to the backs of mice for limited periods of time and only 2 tumors. Subsequent applications of methylcolanthrene or 9,10-dimethyl-1,2-benzanthracene for 2 months raised the incidence to 17 and 15 tumors per group of 20 respectively. Essentially the same results were observed when methylcholanthrene was the precarcinogenic agent. Lavik *et al.* (17) (following paper) have demonstrated that either benzpyrene or dibenzanthracene can complete the carcinogenic process initiated by methylcholanthrene, and that the additive effects of the hydrocarbons are equivalent to their known carcinogenic potencies for mouse skin.

TABLE I: ADDITIVE EFFECT OF CARCINOGENIC HYDROCARBONS *

	Painted		4th week			8th week			12th week			16th week			20th week			28th week		
Group †	1st 8 wks.	2nd 8 wks.	N	P	Ca	N	P	Ca	N	P	Ca	N	P	Ca	N	P	Ca	N	P	Ca
1	DMB	....	20	0	0	20	0	0	19	1	0	17	3	0	17	2	1	12	2	1
2	DMB	DMB	20	0	0	20	0	0	6	14	0	4	16	0	1	12	4	0	4	9
3	DMB	BP	20	0	0	19	0	0	17	2	0	8	9	2	6	9	4	4	7	4
4	DMB	MC	20	0	0	19	0	0	17	2	0	10	8	0	2	11	5	1	2	8
5	BP	....	20	0	0	20	0	0	19	1	0	19	1	0	19	1	0	18	1	1
6	BP	BP	20	0	0	19	0	1	18	0	2	9	7	3	8	6	5	5	4	8
7	BP	DMB	20	0	0	20	0	0	19	1	0	3	11	6	3	8	7	0	8	7
8	BP	MC	20	0	0	19	1	0	18	2	0	8	11	1	5	9	6	2	4	13
9	MC	....	19	1	0	15	5	0	11	9	0	7	13	0	4	10	3	2	8	7
10	MC	MC	18	2	0	15	5	0	10	9	1	2	16	2	0	13	5	0	3	12
11	MC	DMB	20	0	0	16	4	0	10	6	3	0	8	11	0	5	14	Dead		
12	MC	BP	17	3	0	12	8	0	5	14	1	1	15	4	0	6	13	Dead		

* Abbreviations used in this table are:
DMB = 9,10-dimethyl-1,2-benzanthracene .....0.01% in benzene painted 3 times per week.
BP = 3,4-benzpyrene .....0.3% in benzene painted 3 times per week.
MC = methylcholanthrene .....0.3% in benzene painted 2 times per week.
N = negative.
P = papilloma.
Ca = carcinoma.
† 20 mice per group at start.

TABLE II: NONADDITIVE EFFECT OF ULTRAVIOLET IRRADIATION AND METHYLCHOLANTHRENE

Group *	Procedure †	Type of tumor ‡	Per cent of mice with tumors 3 mo.	4 mo.	5 mo.	6 mo.	Total energy Ergs/sq. cm. × 10^7
1	Irradiated 5 mo.	U. V. ray	4	17	52	70	141
2	Irradiated 3 mo.	U. V. ray	0	8	17	25	85
3	Irradiated 3 mo. then	U. V. ray	6	6	8	8	85
	painted 2 mo.	Chemical	0	0	0	9	
4	Painted 2 mo. then	U. V. ray	0	0	0	0	55
	irradiated 2 mo.	Chemical	4	14	33	48	
5	Irradiated 5 mo. and	U. V. ray	0	0	6	10	141
	painted 1st 2 mo.	Chemical	0	5	22	39	
6	Painted 2 mo.	Chemical	4	13	22	41	
7	Painted 4 mo.	Chemical	9	19	34	52	

* 24 mice per group at start.
† Irradiated with 30,000 erg/cm²/sec.; 30 min./day, 6 days/wk. Painted with 0.1% methylcholanthrene in acetone 3 times per week.
‡ U. V. ray = ultraviolet ray.

Invariably, the carcinogenic process initiated by one of the three hydrocarbons could be completed by either of the others. Thus, the application of 9,10-dimethyl-1,2-benzanthracene for 2 months yielded only 3 tumors out of 20 animals. Subsequent treatment with methylcholanthrene or benzpyrene for 2 months raised the incidence to 10 and 11 tumors respectively. Similarly, treatment with benzpyrene for 2 months induced

The combination of ultraviolet irradiation and carcinogenic hydrocarbon.—The hydrocarbon used in the initial experiments was methylcholanthrene, applied to the ears as a 0.1 per cent solution in acetone 3 times weekly. The dosages of hydrocarbon and of ultraviolet light were so chosen that tumor formation

due to either alone proceeded at approximately the same rate, and each carcinogen was applied for only limited periods of time. The various treatments and results are listed in Table II.

When the animals were irradiated for 3 months, no tumors were visible at the end of the irradiation period, but tumors of the ears developed during the subsequent 3 months' period in 25 per cent of the animals. The application of methylcholanthrene for 2 months to ears which had previously been irradiated for 3, not only failed to increase the number of irradiation-induced tumors, but actually reduced the number of ear tumors formed. The treatment with hydrocarbon alone resulted in tumors in 41 per cent of the animals. Thus no evidence of an additive effect between methylcholanthrene and ultraviolet light was obtained in this series. It was usually possible to make a gross distinction between the tumors induced by light and those due to hydrocarbon. The former generally were found in the upper part of the ear, and were smooth; the latter were found at the base of the ear or even at the top of the head, presumably because of drainage of the hydrocarbon solution, and in the early stages were typical keratotic papillomas. Tumors initiated by these two agents are listed separately in the tables.

Essentially the same experiments were performed on a somewhat larger scale with 9,10-dimethyl-1,2-benzanthracene as the carcinogenic hydrocarbon. The various experimental modifications and the results are listed in Table III. Again there was no evidence of any additive effect between the hydrocarbon and the light. The incidence of tumors was relatively low in all groups, and such variations in tumor development which appeared were within the limits of experimental error.

TABLE III: NONADDITIVE EFFECT OF ULTRAVIOLET IRRADIATION AND 9,10-DIMETHYL-1,2-BENZANTHRACENE

Group *	Procedure †	Tumors induced by ‡	12th week §			16th week			20th week			24th week			32nd week		
			N	P	Ca	N	P	Ca	N	P	Ca	N	P	Ca	N	P	Ca
1	Irradiated for 12 weeks	U. V. ray	20	0	0	20	0	0	16	0	2	16	0	2	12	0	4
2	Painted for 12 weeks	Chemical	16	3	0	16	2	1	16	2	1	16	2	1	12	1	2
3	Irradiated and painted for 12 weeks concurrently	U. V. ray	24	0	0	23	0	0	22	0	1	20	0	1	13	0	4
		Chemical		0	0		0	0		0	0		0	0		0	0
4	Irradiated for 12 weeks then painted for 12 weeks	U. V. ray	23	0	0	17	0	1	15	0	2	9	0	4	5	0	7
		Chemical		0	0		2	0		1	1		5	1		4	3
5	Painted for 12 weeks then Irradiated for 12 weeks	U. V. ray	18	0	0	14	0	0	11	0	0	7	0	0	3	0	5
		Chemical		0	0		4	0		3	0		5	2		2	3
6	Irradiated for 12 weeks, Rest 4 weeks then Painted for 12 weeks	U. V. ray	24	0	0	23	0	0	22	0	0	20	0	0	6	0	5
		Chemical		0	0		0	0		0	0		1	0		3	2
7	Painted for 12 weeks, Rest 4 weeks then Irradiated for 12 weeks	U. V. ray	24	0	0	19	0	0	16	0	0	12	0	3	5	0	5
		Chemical		0	0		4	1		5	1		4	1		2	1
8	Irradiated and "scatter painted" for 12 weeks concurrently	U. V. ray	19	0	1	19	0	1	19	0	1	16	0	2	13	0	3
		Chemical		0	0		0	0		0	0		0	0		0	0

* 24 mice per group at start.
† Irradiated with 30,000 erg/cm²/sec. for 30 min./day, 6 days/week.
Painted with 9,10-dimethyl-1,2-benzanthracene (0.01% in benzene) 3 times per week.
"Scatter painting" was done by applying hydrocarbon to sides, abdomen, back, legs, and tail on alternate times.
‡ U. V. ray = ultraviolet ray.
§ N = negative.
P = papilloma.
Ca = carcinoma or sarcoma.

The effect of concurrent applications of hydrocarbon and ultraviolet light was also tested on mice. In one experiment a solution of methylcholanthrene was applied to the ear 3 times weekly immediately before irradiation and fewer irradiation-induced tumors resulted (Table II, group 5). This result was first attributed to an absorption of the carcinogenic wave lengths by the hydrocarbon. However, when the hydrocarbon was applied to ears immediately after the raying period rather than prior to it, the development of irradiation tumors was still inhibited. Apparently, therefore, the inhibition was due to something more fundamental than a screening effect. Conversely, it was also of interest to determine the influence of ultraviolet irradiation on hydrocarbons. The photolability of dibenzanthracene has been demon-

strated by Boyland (7) who obtained a water-soluble derivative which inhibited lactic dehydrogenase. Accordingly, we have prepared an analogous fraction from methylcholanthrene and tested it for carcinogenicity. The hydrocarbon was dissolved in benzene, spread over a large area, and irradiated with a mercury vapor lamp for 3 hours at a distance of 10 cm. The benzene evaporated quickly. Some of the hydrocarbon turned brown and became soluble in dilute NaOH, and this material was applied to the skin of 48 mice over a 7-month period. None of the mice thus treated developed cancer.

An attempt was therefore made to determine whether the hydrocarbon exerted a systemic influence which might alter carcinogenesis with ultraviolet light. A solution of 9,10-dimethyl-1,2-benzanthracene was applied alternately to the back, sides, abdomen, legs, and tail of mice being irradiated. Thus relatively large amounts of carcinogen could be administered without danger of inducing cancer in any particular area. Such treatment, however, had no effect on irradiation tumors (Table III, group 8). The subcutaneous injection of 0.5 mgm. of methylcholanthrene, or of 1.5 mgm. of dibenzanthracene in corn oil into irradiated mice markedly impaired their physical condition, and many of the mice died of subcutaneous sarcomas before tumors of the ear due to the light developed. No direct effect on the formation of irradiation tumors could be ascribed to the injected hydrocarbons.

The combination of noncarcinogens and ultraviolet irradiation.—Several workers have demonstrated that the formation of tumors induced by chemical carcinogens can be inhibited or accelerated by agents which themselves are noncarcinogenic (5, 9, 26). Similar modifications might be expected for tumors induced by ultraviolet light, and photosensitizing agents, in particular, might be expected to accelerate the development of irradiation tumors. The various substances tested are listed in Table IV. A detailed account of the results has been omitted, since the substances tested, with the single exception of estrone, failed to alter the development of irradiation tumors. The mice injected with estrone were in poor physical condition, most of them lost weight, and many died with enlarged bladders. Tumor development was inhibited in this group. It is possible that cocarcinogenic effects might be demonstrated for some of the substances under different experimental conditions; *e.g.*, there is the possibility that cancer might be produced with noncarcinogenic wave lengths of light provided suitable chemicals were present to absorb the energy.

TABLE IV: SUBSTANCES WHICH DID NOT ALTER CARCINOGENESIS WITH ULTRAVIOLET IRRADIATION

Compound *	Concentration and solvent	Total amount given, in mgm.	Method of administration	Number of times administered per week
Rose bengal	42% in alcohol	....	Paint on ears	2
Rose bengal	0.2 mgm. in water	10.0	Subcutaneously in back	2
Hematoporphyrin	0.25-0.5 γ in water	0.015	Subcutaneously in ears	2
Methylene blue	0.2 mgm. in water	9.4	Subcutaneously in back	2
Trypan blue	0.2 mgm. in water	9.4	Subcutaneously in back	2
Vital red	0.2 mgm. in water	9.4	Subcutaneously in back	2
Neutral red	0.1% in benzyl alcohol		Paint on ears	2
Dichlorophenol-indophenol	0.1% in benzyl alcohol		Paint on ears	6
Histamine	0.1% in benzyl alcohol		Paint on ears	6
Benzyl alcohol	100%		Paint on ears	6
Catechol	1% in benzene		Paint on ears	2
p-Thiocresol	0.5% in benzene		Paint on ears	2
Benzene	100%		Paint on ears	2
Testosterone propionate	0.25 mgm. in oil	8.0	Subcutaneously in back	2
Estrone	125 I.U. in oil	3,125 I.U.	Subcutaneously in back	2

* 24 mice per group for each compound. Irradiated with 30,000 erg/cm²/sec. 30 min. daily 6 days/week for 6 months. The chemicals were administered during the period of irradiation.

The combination of Shope papilloma virus and ultraviolet or x-irradiation.—The additive effect of tar or methylcholanthrene and the papilloma virus has been clearly demonstrated by Rous and associates (18, 22). Such summation might result from the localization of the virus by the application of the chemical although other combination effects must also be considered. It was therefore of interest to determine whether ultraviolet light could replace hydrocarbon in similar experiments. Forty-eight young adult albino female rabbits were used, and 12 of these were exposed to ultraviolet light for 1 hour daily for 2 months. An average of 30,000 ergs/cm²/sec. reached the ears, although this varied greatly with the position in which the rabbit held its ears. Since the rest of the animal was protected against the light by its fur, the procedure was deemed the equivalent of irradiating the ears only. After 2 months, the raying was discontinued and 10 cc. of a clear saline suspension of the Shope papilloma virus, prepared as pre-

viously described (4), was injected into the ear veins of 6 of the rabbits. A similar injection was given 8 rabbits which had received no previous irradiation. A few large warts developed on the ears at the points of injection and several small papillomas were found scattered not only on the ears but also on other areas of the skin. The size and number of the virus nodules were essentially equal in irradiated and unirradiated rabbits. No tumors developed as a result of the irradiation alone, for the intensity was essentially that which failed to produce tumors in rabbits after 11 months of exposure (4). The amount of energy, however, was more than adequate for the production of tumors in rats and mice.

Sixteen rabbits were treated with a single dose of 2,000 r of x-ray to the distal 8 cm. of the left ear. Two days later a clear suspension of the Shope papilloma was given intravenously into a leg vein of 8 of the rabbits while the remaining 8 were given a similar injection 10 days after the x-ray treatment. Twelve rabbits were injected with the virus only. Again, small warts occurred on the ears of some of the rabbits in both the irradiated and nonirradiated groups. It was possible that x-irradiation had inhibited papilloma formation somewhat when administered 2 days prior to the injection of virus and that it had stimulated tumors slightly when the virus was given 10 days after the exposure of x-ray, but this was not conclusive.

DISCUSSION

When two different carcinogenic hydrocarbons were applied to the same area of skin for separate periods, the carcinogenic effects were summated, the skin apparently being unable to differentiate between the two chemicals and responding as if one carcinogen were acting all the time. It would appear therefore that the mechanism of carcinogenesis by these two agents is identical, and conversely that the failure of ultraviolet irradiation and the hydrocarbon to reinforce one another might be attributed to differences in the carcinogenic mechanisms involved. It should be pointed out, however, that in the mouse, irradiation tumors generally arise from connective tissue elements (12) whereas the application of hydrocarbons to the skin results in epithelial neoplasms, although each agent by itself may affect both kinds of tissue, since epithelial tumors due to ultraviolet light are not infrequent (21, 23) and hydrocarbons readily induce sarcomas under the proper experimental procedure. It has been suggested that there may be two or more distinct steps in the carcinogenic process and the terms pre-, epi-, and metacarcinogens have been applied to agents involved in one or more of these phases (5). Carcinogens are not always needed to complete the carcinogenic process since substances such as croton oil resin (5) and scalding water (9) will produce tumors in tissues previously rendered precancerous. Thus the lack of summation between irradiation and virus or hydrocarbon would suggest that the light lacks precarcinogenic activity with respect to these agents.

Several theories have been proposed for the mode of action of ultraviolet light. Thus it has been suggested that radiant energy converts cholesterol or some other naturally occurring substance into a carcinogenic chemical. This, however, appears unlikely in view of the absence of additive effects between light and hydrocarbons, in contrast to the consistent additive effects observed between the carcinogenic hydrocarbons themselves. Furthermore, benzene extracts of irradiated skin or of irradiation tumors have repeatedly failed to induce tumors when injected subcutaneously in corn oil, and irradiated cholesterol, also, was noncarcinogenic in our experiments,[2] an observation made also by Bergmann and associates (6).

It has been suggested that ultraviolet light initiates cancer by releasing an "indigenous virus." Lambeau (15), for example, has shown that a latent herpes zoster virus may be activated and localized by treatment with ultraviolet light. The Shope papilloma virus, however, did not have an enhanced effect on irradiated skin. A third explanation of the carcinogenic effect of ultraviolet light involves a direct action of the light on cellular proteins. The carcinogenic wave lengths lie between 2,900 and 3,300 A. (25) and it is well known that many proteins and nucleoproteins absorb light of these wave lengths. A disturbance in the nucleoprotein or chromatin of the cell might well cause abnormal cell division. Ultraviolet irradiation is one of the most commonly used procedures for inducing changes in the chromosomes of germ cells and Mackenzie and Muller (19) have recently shown that the wave lengths producing mutations in *Drosophila* range from 3,000 to 3,200 A. Thus a similarity might be suspected in the mechanisms for the production of both mutations and tumors by these wave lengths. The possible role of the altered protein in carcinogenesis is discussed in the following paper (17).

SUMMARY AND CONCLUSIONS

1. The carcinogenic process initiated by the application of benzpyrene, methylcholanthrene, or 9,10-dimethyl-1,2-benzanthracene to the backs of mice could be completed by either of the other two hydrocarbons.

2. Ultraviolet light did not increase the number of tumors produced in mice rendered precarcinogenic with methylcholanthrene or 9,10-dimethyl-1,2-benzan-

[2] Unpublished data.

thracene, and the hydrocarbons failed to increase the number of tumors in animals which where precarcinogenic with respect to irradiation tumors. Certain hormones, vital dyes, and other substances, likewise failed to increase the number of tumors induced by ultraviolet irradiation.

3. Ultraviolet irradiation and x-rays both failed to increase the carcinogenicity of the Shope papilloma virus.

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Some Additive Effects of Carcinogenic Hydrocarbons*†

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The present study deals with the additive effects of different carcinogenic hydrocarbons and with the effect of small amounts of carcinogen on mouse skin many months after the tissue had been rendered precancerous by treatment with methylcholanthrene.

MATERIALS AND METHODS

Delayed Development of Tumors

Table I contains a summary of all experiments in which solutions of 20-methylcholanthrene were applied to the backs of mice for 2 months. At the end of this period 2 out of 282 mice on the control diet (9) and 49 of 838 on various diets high in fat had developed tumors. Although treatment was suspended, tumors continued to appear in large numbers during the 3rd month, with a decreasing number of new tumors appearing each subsequent month. The rate of growth of a tumor is essentially the same whether it had been induced rapidly or slowly (14). Hence, the delayed appearance of many of the tumors in the present series suggested a corresponding delay in their induction; *i.e.*, a continuation of the carcinogenic process long after the applications of hydrocarbon were discontinued.

Table I: Progressive Development of Tumors as a Result of the Application of 0.2 to 0.3 Per Cent Methylcholanthrene for 2 Months

Period, in months	Tumors developed on control diet		Tumors developed on high fat diets	
	Number	Per cent	Number	Per cent
0-2	2	3.0	49	9.4
2-3	33	49.3	302	58.2
3-4	18	27.0	124	24.0
4-6	14	21.0	46	8.4
Totals	67	100.0	519	100.0
Effective total	282		838	

Additive Effects of Carcinogenic Hydrocarbons

Hieger (7) observed that one carcinogen may complete the intracellular reactions begun by another, when tumors developed in 2 of 10 mice which were painted with 3,4-benzpyrene for 8 weeks, followed by similar treatment with 1,2,5,6-dibenzanthracene. Each treatment by itself failed to produce tumors. In view of these fragmentary data we have studied the additive effects of hydrocarbons in some detail.

In a preliminary experiment 20 heterozygous albino mice were painted on the interscapular region with a 0.2 per cent solution of methylcholanthrene in dioxane twice weekly for 2½ months. A comparable group of animals was painted with methylcholanthrene during the 1st month, with a 0.2 per cent solution of benzpyrene during the 2nd month, and with methylcholanthrene during the final one-half month. The incidence of tumors in the two groups was equal, and the rate of tumor formation was also essentially the same.

A comparison was next made of the abilities of various hydrocarbons to increase the rate of tumor formation in animals which had already received subcarcinogenic amounts of methylcholanthrene. Four groups of 25 young adult mice were used. The initial treatment consisted of the application of a 0.3 per cent solution of methylcholanthrene in dioxane for 2 months. The first group received no additional treatment. The other groups were painted with a 0.3 per cent solution of dibenzanthracene, benzpyrene, or methylcholanthrene twice weekly during the 3rd month. The results are summarized in Table II. The additional application of hydrocarbon increased the rate of tumor formation markedly, and the effect of each hydrocarbon during the 3rd month was proportional to its known carcinogenic potency when applied

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throughout the period of carcinogenesis. Thus dibenzanthracene increased tumor incidence from 17 to only 37 per cent at 4 months. Benzpyrene increased the incidence to 74 per cent, whereas methylcholanthrene increased it to 91 per cent during the same period.

These results were confirmed in a second series in which more dilute solutions were employed. Methylcholanthrene was applied as a 0.2 per cent solution in dioxane twice weekly for 2 months, followed by the application of similar solutions of benzanthracene, dibenzanthracene, benzpyrene, or methylcholanthrene for an additional month. Applications of benzpyrene and methylcholanthrene were also combined in certain other ways (Table II). Benzanthracene failed to increase tumor formation, but the other hydrocarbons increased the number of tumors in proportion to their known carcinogenic potencies for mouse skin.

Table II: Additive Carcinogenic Effects of Various Hydrocarbons on the Skin of Albino Mice

Carcinogenic treatment *	Percentage of mice with tumors at various months								Effective total
	2½	3	3½	4	4½	5	5½	6	
0.3% MC, 2 months	4	4	12	17	17	17	17	17	24
0.3% MC, 2 months, 0.3% DBA 3rd month	12	33	33	37	46	46	50	58	24
0.3% MC, 2 months, 0.3% BP 3rd month	16	53	68	74	79	84	84	84	19
0.3% MC, first 2 months, 0.3% MC 3rd month	9	52	78	91	100	..	.		23
0.2% MC, 2 months	0	4	4	4	4	9	13	18	22
0.2% MC, 2 months, 0.2% BA 3rd month	0	4	9	14	14	14	14	14	22
0.2% MC, 2 months, 0.2% DBA 3rd month	4	9	27	32	36	36	36	41	22
0.2% MC, 2 months, 0.2% BP 3rd month	0	13	23	50	55	55	55	55	22
0.2% MC, first 2 months, 0.2% MC 3rd month	4	4	17	39	57	84	96	100	23
0.2% BP, 1st month, 0.2% MC 2nd month, 0.2% BP 3rd month	0	0	15	35	40	45	45	53	20
0.2% BP, and 0.2% MC given alternately by the week for 3 months	0	5	5	14	19	24	29	29	21
0.1% BP, 3 months, 0.1% MC 3 months	0	15	25	60	70	75	75	75	23

* MC = 20-methylcholanthrene.
DBA = 1,2,5,6-dibenzanthracene.
BP = 3,4-benzpyrene.
BA = 1,2-benzanthracene.

While the additive effects of the carcinogens varied somewhat with the mode of administration (Table II), the results in general indicated that dibenzanthracene and benzpyrene, as well as methylcholanthrene, stimulated the production of tumors in tissues rendered precarcinogenic with methylcholanthrene. Consistent additive effects of different hydrocarbons have also been observed by Rusch *et al.* (12) who used 9,10-dimethyl-1,2-benzanthracene in combination with other carcinogenic procedures.

Tumor Developmont in Precancerous Mouse Skin

Preliminary experiments were conducted as follows: Large numbers of mice receiving various diets (9) were painted with 0.3 per cent solutions of methylcholanthrene in dioxane twice weekly for 2 months. Seventy of the animals which failed to develop tumors 3 or 4 months after the last application of hydrocarbon were then repainted with methylcholanthrene twice weekly for 1 month. Our ordinary stock diet was fed during the repainting period and thereafter until the end of the experiment. The second treatment was by itself inadequate for tumor production, and the original application of hydrocarbon had been sufficiently mild to permit a complete regrowth of hair in the interval between the two periods of application. Furthermore enough time had elapsed so that very few new tumors were appearing as a result of the original treatment (Table I). Nevertheless, over 50 per cent of the repainted animals developed tumors.

The experiment was repeated with 5 groups of 15 to 20 animals each. The precarcinogenic treatment consisted in the application of either 0.2 or 0.3 per cent solutions of methylcholanthrene and resulted in a tumor incidence of 15 to 25 per cent. The interval between applications was 3 or 4 months, and the second treatment consisted in the application of 0.3 per cent methylcholanthrene or benzpyrene in dioxane twice weekly for 1 month. This latter produced a much more severe response than that observed in animals receiving methylcholanthrene for the first time. Marked alopecia, thickening of the skin, soreness, and scabbiness appeared in practically all of the repainted animals. In fact these lesions were frequently so severe that it was difficult to determine when tumors first appeared, but new tumors continued to develop for 4 months after the second repainting treatment was discontinued (Fig. 1). The final incidence of tumors exceeded 50 per cent of the effective totals based upon the appearance of the first tumor after the repainting had been begun. The effectiveness of the repainting

treatments did not appear to depend upon the character of the diet fed originally, nor upon the daily application of benzene during the repainting period. Benzpyrene was nearly as effective as methylcholanthrene in inducing tumors under these conditions.

During the repainting period the application of methylcholanthrene was very much more effective in promoting the development of tumors than various "cocarcinogenic procedures" attempted (Table III). Tumor incidence was not increased by the feeding of a diet containing 15 per cent of fat, nor by an incision ½ inch long in the painted area of the backs of precarcinogenic animals. A slight increase in tumors resulted when benzanthracene was painted for 5 months after the start of the second period. In contrast, the application of methylcholanthrene for only

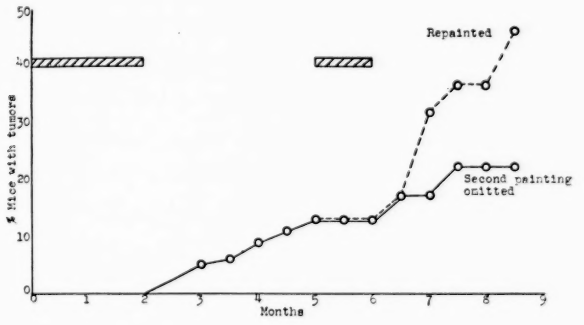

FIG. 1.—Increased tumor formation due to a renewed application of hydrocarbon after a 3-month interval. The cross-hatched areas represent the periods of application of methylcholanthrene.

1 month increased the incidence of tumors to 70 per cent. This "secondary" treatment with methylcholanthrene failed to produce any tumors in 29 mice of similar age which had received no original treatment with methylcholanthrene.

DISCUSSION

The observed additive effects of the carcinogens indicate that the original treatment of the skin with methylcholanthrene had produced changes which persisted for many months and which were capable of ultimately contributing to the formation of tumors. Similar observations have been made in mice by Beck and Peacock (2) and in rabbits by Rous and Kidd (11). Two explanations might be offered. The first is that tumor cells had been formed during the original treatment but that they lay dormant until activated by hydrocarbons or other agents (3, 4, 13). This explanation appears to be favored by MacKenzie and Rous (10). A second explanation could be that the change induced by the original treatment was literally subcarcinogenic; *i.e.*, that no tumor cells were present as such but only precancerous or "part-tumor" cells.

TABLE III: TUMOR FORMATION IN PRECANCEROUS ANIMALS

Group	Initial treatment		Weeks since last painting	Second treatment		Number of mice per group	Effective total	Percentage tumor formation—months								
	Diet	Carcinogen		Diet	Carcinogen			1	1½	2	2½	3	3½	4	4½	5
1	Stock	None	..	Grain diet	0.3% MC, 1 month	29	..	0	0	0	0	0	0	0	0	0
2	Grain diet	0.3% MC,* 1 month	12	Grain diet	None	16	15	0	6	6	13	13	13	13	13	18
3	Grain diet	0.3% MC,* 1 month	12	Grain diet	0.3% MC, 1 month	16	10	0	10	40	50	50	70	..	..	..
4	Grain diet	0.3% MC,* 1 month	12	Grain diet	0.3% BA† duration	17	14	0	14	21	21	28	35	35	35	35
5	Grain diet	0.3% MC,* 1 month	12	Grain diet	None: incision	17	14	7	14	14	14	14	14	14	14	21
6	Grain diet	0.3% MC,* 1 month	12	15% fat	None	17	16	0	12	12	12	12	12	12	12	12
7	Stock	Mixed painting	14	Stock	0.3% MC, 1 month	24	19	15	42	47	58	74	74	..	..	..
8	Stock	Mixed painting	14	Stock	None	12	..	0	0	0	0	0	0	..	..	..

* MC = 20-methylcholanthrene.
† BA = 1,2-benzanthracene.

These can be visualized, if it is assumed that the significant change in carcinogenesis is an alteration in tissue protein, possibly in nucleoprotein or chromatin.

Beck (1) has stated that benzpyrene is "titrated automatically against the tissues in the course of its absorption," and that this process determines which of the susceptible cells shall become malignant. Fieser (6) has suggested that the hydrocarbon exerts a modifying influence on a cell constituent, and further that "the postulated interaction is best conceived as occurring between the carcinogen and an intact proteinoid constituent of the cell protoplasm." While the altered hydrocarbon can be studied experimentally, it would appear that the more significant product of the "titration" is the altered protein. The alteration must be sufficiently mild, however, so that the cell can still use the new substance as a model for its subsequent synthesis or reproduction by the cell itself. Once enough altered or "tumor protein" is accumulated within the cell, tumor characteristics appear; if insufficient amounts of tumor protein are present, the cell is a "part-tumor" cell.

Kögl (8) has pointed out the connection between the latent period in carcinogenesis and the time necessary for the turnover or complete resynthesis of tissue protein, but unfortunately he has emphasized specific configurative changes within amino acids, rather than changes in protein as such. His more general considerations are, however, applicable to carcinogenesis by the hydrocarbons. If the new protein were not synthesized any faster than the old, then tumors would fail to appear unless enough additional carcinogen were added to increase the concentration of tumor protein above the critical level. If the rate of synthesis of the new protein were slightly greater than that of the old, then the concentration of new protein could increase until that level was reached at which tumor characteristics appear, and the over-all result would be a delayed development of tumors such as we have observed. Recently Cramer and Stowell (5) applied single doses of methylcholanthrene to the skin of mice at intervals of several weeks, and observed that much less hydrocarbon was needed for tumor formation when the interval between paintings was large. This would necessarily follow, if the new protein increased in the intervals between applications of hydrocarbon.

SUMMARY AND CONCLUSIONS

1. Of 586 mice which developed tumors as a result of the application of methylcholanthrene for 2 months, 92 per cent did not develop them until after the carcinogenic treatment had been discontinued.

2. Methylcholanthrene was applied to the skin of mice in amounts insufficient to produce tumors in the majority of the animals, and methylcholanthrene, benzpyrene, dibenzanthracene, or benzanthracene were then applied for relatively short periods of time. The hydrocarbons exerted additive effects which were equivalent to their known carcinogenic potencies for mouse skin.

3. When methylcholanthrene was applied to the skin of mice for 2 months and intervals up to 4 months allowed before the application of hydrocarbon was resumed for 1 month more, many tumors developed as a result of the repainting procedure.

4. These observations suggest that the initial application of hydrocarbon had produced subcarcinogenic alterations in the tissue. An attempt has been made to correlate the results with an hypothesis that carcinogenesis is essentially an accumulation of abnormal protein within the cell.

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The Effect of Solvents in Methylcholanthrene Epidermal Carcinogenesis*

A Comparison of Benzene and Acetone

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The application of carcinogenic hydrocarbons to the skin of experimental animals has been the subject of numerous investigations since Cook and Kennaway and their associates first used dibenzanthracene in 1930. However, few observations have been published on the effect of the method of application or of the solvent for the carcinogen, upon carcinogenesis. In other publications (6-8) we have emphasized the importance of variations in the technic of the application of methylcholanthrene, such as the frequency of painting, the size of the exposed area, and the concentration of the carcinogen. In this paper, which is a contribution to a group investigation directed by Dr. E. V. Cowdry, benzene and acetone are compared as solvents for 20-methylcholanthrene in epidermal carcinogenesis.

The importance of solvents in the production of sarcoma by the subcutaneous injection of methylcholanthrene has been discussed by others, including Shimkin and Andervont (12).

Benzene and chloroform have been the most commonly used solvents for the topical application of carcinogens. In 1937, Taschner, Gottleib, and Spritzer (14) used acetone as a solvent for methylcholanthrene. They found it less toxic than benzene for the mice. Berenblum (2) and Bachmann and his associates (1) used acetone as the solvent for various hydrocarbons applied to the skin of mice. For his study of changes antecedent to tumor formation, Orr (9) preferred acetone to benzene as a solvent for carcinogenic hydrocarbons because acetone had less effect upon the skin. Pullinger (10) also stated a preference for acetone as a solvent, but none of these investigators published comparative observations on the effect of solvents upon carcinogenesis. When Crabtree (5), to retain the carcinogen in a film at the site of application, added 2 per cent liquid paraffin to acetone, ether, or benzene, the production of benign tumors in the skin of mice was accelerated. He used several different carcinogens in his series and did not directly compare the solvents acetone and benzene. But, as Watson (15) and subsequently Bonser (4) have shown, the more rapid production of benign epidermal tumors under one set of conditions does not necessarily indicate an earlier production of malignant tumors. Quite recently Bradbury, Bachmann, and Lewisohn (3), using chiefly small groups of 15 or less mice, reported that acetone had a definite accelerating effect on the action of certain carcinogenic agents when applied to the skin. They attribute this to the water-miscible property of acetone.

* This investigation was aided by a grant from an anonymous donor.

MATERIALS, METHODS, AND RESULTS

Female Swiss mice 3 months of age were kept under similar environmental conditions in three series which were run simultaneously. In the "benzene series" (series I) 39 mice were painted thrice weekly with a 0.3 per cent solution of methylcholanthrene in benzene. In the "acetone series" (series II) a 0.3 per cent solution of methylcholanthrene in acetone was applied to 39 mice 3 times a week. The 45 mice in series III—the "acetone-benzene series"—were painted thrice weekly with the same 0.3 per cent solution of methylcholanthrene in acetone on the same days as the animals in series I and II and, in addition, on alternate days, 3 times a week, they were painted with a solution of the pure benzene solvent. The benzene used was of Analytical Reagent grade and the acetone of U.S.P. quality. Because of its low solubility it is not feasible to use a solution of a higher concentration than 0.3 per cent of methylcholanthrene in acetone. When not in use all solutions were kept tightly stoppered in the icebox to reduce evaporation of the solvent and changes in concentration of the solute.

Using a standard technic described in a previous paper (6, 7), the various solutions were applied by the same worker to a moderately large area of the back of each mouse with a single stroke of a No. 4 camel's hair brush. All mice received 42 applications in a period of 100 days, after which time no additional treatment was given. The mice gained in weight, suggesting that the painted solutions were well tolerated. Macroscopic observations on the presence of epilation, ulceration, swellings, papillomas, and malignant neoplasms were recorded each week. In each mouse the presence of a malignant neoplasm was confirmed by microscopic examination of several sections taken at different angles through the skin of the mouse's back.

At the end of the 1st week of the experiment 85 per cent of the mice in the benzene series, 78 per cent in acetone-benzene series, and 64 per cent of the animals in the acetone series showed some degree of epilation.

By the 3rd week the hair had started to regrow in many of the animals so that fewer mice showed complete epilation; however, practically all of the mice in each series showed some epilation.

On the basis of our previous experiments, tumors were classified as elongated swellings, warty swellings, papillomas, or malignant tumors. The elongated swellings appeared near the midline of the back as one or two ridges in the skin parallel to the long axis of the mouse and to the direction in which the brush with its solution was applied. These elongated, edematous-appearing swellings often disappeared rather suddenly or they were sometimes transformed into warty swellings or into true papillomas. The warty swellings were more discrete, semiglobular elevations in the skin, and they either regressed or developed into papillomas.

The incidence of elongated swellings, which was similar in each series, increased rapidly up to the 3rd week after which it began to decrease. The warty swellings, a nonneoplastic type of tumor which often developed eventually into papillomas, increased steadily in incidence after the 2nd week for several weeks. At the 3rd week 77 per cent of the mice in the acetone series, 67 per cent of the mice in the acetone-benzene, and 56 per cent of those in the benzene series had either elongated or warty swellings on the painted skin area. The production of swellings was slightly slower in the benzene series which did not reach its maximum of 67 per cent until the 4th week of the experiment.

Papillomas are usually larger than the warty swellings and, in contrast to the swellings, represent a type of tumor which very rarely regresses while an animal is being painted with the carcinogen. The first papillomas in the benzene and in the acetone-benzene series appeared in the 4th week and in the acetone series in the 6th week.

However, it is the comparative incidence of malignant neoplasms in the three series, as shown in Fig. 1, which is most significant. The first two carcinomas appeared after 6 weeks in the acetone-benzene series. This group of animals, which was treated with methylcholanthrene in acetone and on alternate days with pure benzene, showed the highest incidence of cancer throughout the experiment. The next highest incidence of carcinomas was among the mice in the acetone series, which were treated with methylcholanthrene in acetone. The least effective results were obtained in the benzene series using methylcholanthrene in benzene. For instance, by the 16th week of the experiment 75 per cent of the mice in the acetone-benzene series, 59 per cent of those in the acetone series, and 43 per cent of the mice in the benzene series had developed cancer. One mouse in the benzene series which was killed in the 13th week of the experiment had not yet developed a carcinoma and, although this mouse might have soon developed a malignant tumor, it was counted as a negative animal in the data. The experiment terminated with malignant neoplasms in all of the surviving mice at about the same time in all three series; *i.e.*, 29 to 31 weeks after the first application of carcinogen. Naturally, the differences in the total incidence of carcinomas in the various series becomes less striking as the experiment approaches its conclusion when all of the mice develop cancer.

In the three series 30 per cent of the mice had 2 carcinomas and an additional 15 per cent had a total of 3 or 4 carcinomas. One mouse in each series had a lymph node metastasis and one in the acetone-benzene series had leukemia in addition to skin carcinoma. Several mice in each series, a total of 11 per cent in the three series, had sarcomas in the area exposed to the carcinogen in addition to carcinomas of the skin. One mouse in the acetone series had only a sarcoma and no carcinoma. The incidence of ulceration in the three series was similar. Of the total of 74 per cent of the mice which developed ulceration of the skin during the experiment, the ulceration in 88 per cent of these occurred during the time the mice were being treated with methylcholanthrene. Ulceration was observed during the period of painting and preceding the diagnosis of a malignant neoplasm in only two-fifths of the 15 mice developing sarcoma.

One animal in the group painted with methylcholanthrene in benzene, which was killed in the 29th week of the experiment, had heterotopic bone in the skin adjacent to an anaplastic carcinoma. A detailed report (13) has been made of this and of three other cases of osteosis cutis which occurred in another experiment.

Several experiments were performed to study the early changes produced in the mouse's skin by solutions of methylcholanthrene and of acetone and benzene. Details of observations on the effects of a 0.6 per cent solution of methylcholanthrene in benzene after single and repeated applications to the skin of the backs of mice have already been reported (7, 8). We found the initial effect of the carcinogen upon the skin to be a toxic one reaching its maximum at about the 3rd day after the first painting, at which time there was a destruction of the epithelial cells in the central portion of the painted area surrounded by a peripheral zone of differentiated epidermis of increased thickness. In contrast to the normal mouse epidermis which is about two cells thick, this differentiated epidermis more clearly resembles normal human epidermis and contains basal, spinous, and granular layers and an increased amount of keratin. By the end of the first week the destructive effect was diminished and the peripheral area of skin had increased further in thickness. By the end of the second week the central portion of the painted epidermis also became differentiated.

Similar results were observed in groups of mice painted with a 0.3 per cent solution of methylcholanthrene in either acetone or benzene. Following a single application of this solution the destructive effect was similar but somewhat less pronounced, and the epithelium showed a similar degree of differentiation. With repeated paintings of the acetone solution of the carcinogen, there was less dilation of the blood vessels in the early stages and the lymphatics, especially those around the hair follicles, appeared to be more dilated.

The effect of the pure benzene and acetone solvents upon the mouse skin was also studied. Two mice were killed on both the 3rd and 5th days following a single application of solutions of benzene and of acetone. Although with benzene little change was visible in the microscopic appearance of the skin on the 3rd day, by the 5th day the epidermis had increased in thickness and become differentiated. In some areas there was also a rather diffuse infiltration of the dermis by inflammatory cells, chiefly polymorphonuclear neutrophils and macrophages. On the other hand, following a single painting with the acetone solvent no evident changes were seen in the skin after 3 or 5 days.

To study the vasodilatory effect of methylcholanthrene and of the acetone and benzene solvents, various solutions were painted on localized areas of the ears of a rabbit. The results after a single application are summarized in Table I, in which the intensity of the hyperemia is indicated by the number of plus signs. The single application of a 0.6 per cent solution of methylcholanthrene in benzene produced an intense hyperemia of the skin extending slightly beyond the periphery of the painted area which lasted 6 days. The hyperemia from pure benzene alone was less pronounced and of briefer duration. The vasodilation in the rabbit's ear produced by a single application of a 0.3 per cent solution of methylcholanthrene in acetone, which represents virtually a saturated solution, is slower in onset, and of about 5 days' duration. Pure acetone, on the other hand, did not produce any appreciable hyperemia of the skin. With several repeated applications of 0.3 per cent solutions of methylcholanthrene in benzene and in acetone to the rabbit's ear, the hyperemia after the second application persisted several days longer than after a single painting. After the 3rd and 4th paintings the vasodilation was less pronounced and of briefer duration.

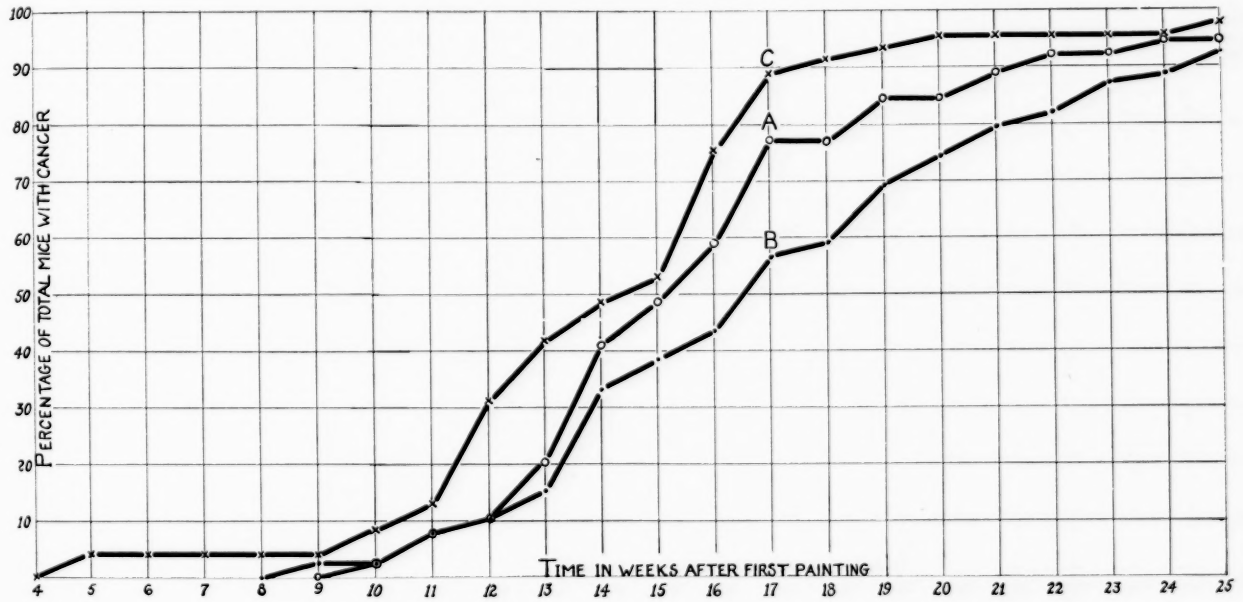

FIG. 1.—Incidence of malignant neoplasms of the skin during the first 26 weeks of the experiment, expressed in percentages of the total number of animals. Mice in the various series were painted on a moderately large area of the back (A) with a 0.3 per cent solution of methylcholanthrene in acetone, thrice weekly; (B) with a 0.3 per cent solution of methylcholanthrene in benzene, 3 times a week; and (C) with a 0.3 per cent solution of methylcholanthrene in acetone and on alternate days, 3 times a week, with pure benzene.

TABLE I: DEGREE OF HYPEREMIA INDUCED IN RABBIT'S EAR BY A SINGLE PAINTING OF VARIOUS SOLUTIONS

Days after application	0.6 Per cent methylcholanthrene in benzene	Pure benzene	0.3 Per cent methylcholanthrene in acetone	Pure acetone
1	++	++	—	—
2	+++	+++	+	—
3	+++	++	+++	—
4	+++	+	+++	—
5	+++	+	++	—
6	++	—	+	—
7	—	—	—	—

DISCUSSION

The results shown in Fig. 1 indicate that the carcinogenic response to a 0.3 per cent solution of methylcholanthrene is better in acetone than in benzene solvent. These observations are in agreement with those of Bradbury, Bachmann, and Lewisohn whose series of mice were much smaller than ours.

The efficacy of the carcinogenic response of epidermis to methylcholanthrene is usually analyzed by criteria relating to time, such as the incidence of cancer in the first animal, in 50 per cent of the group, and in 100 per cent of the animals. Differences of only a few weeks in these criteria, especially when small numbers of experimental animals are involved, may not be significant. In experiments designed to achieve a maximum carcinogenic response in a relatively short period of time, a more significant criterion may be the percentage of malignant tumors occurring during a period of several weeks of the experiment.

In a group of 27 Swiss mice painted with a 0.6 per cent solution of methylcholanthrene in benzene, in which the carcinogen is much more soluble, the first carcinoma appeared in 6 weeks and by the 24th week all the animals had developed malignant tumors (7). Although the 0.6 per cent solution of methylcholanthrene in benzene produced carcinoma earlier in both the most susceptible and in the most resistant animals of the group, the incidence of malignant tumors during selected periods of several weeks was higher with the 0.3 per cent solution in acetone. Between the 12th and 17th week of the experiments 67 per cent of the mice painted with 0.3 per cent methylcholanthrene in acetone, 46 per cent of the mice painted with 0.3 per cent methylcholanthrene in benzene, and 41 per cent of the mice painted with the 0.6 per cent methylcholanthrene in benzene developed carcinomas. Actually, a better carcinogenic response to the 0.6 per cent solution was obtained at a later period, between the 14th and 19th weeks of the experiment, when 59 per cent of the mice developed malignant neoplasms.

With our present information, it is difficult to explain the effect of solvents upon the process of epidermal carcinogenesis. Certain substances providing a specific type of trauma for the cells—the cocarcinogens of Sall and Shear (11) and of Berenblum (2)—seem to supplement the trauma of the methylcholanthrene and facilitate the more rapid production of cancer. In studying the mode of action of methylcholanthrene, the use of such cocarcinogens would complicate the problem. At present it is not possible to say whether the more rapid production of malignant neoplasms in the acetone-benzene series is due (a) to the redissolving of carcinogen by the application of pure benzene on alternate days, thus giving these mice the near-equivalent of daily paintings with methylcholanthrene; (b) to a cocarcinogenic action of the solvent benzene; or (c) to some other factor.

SUMMARY AND CONCLUSIONS

Epidermal carcinogenesis was obtained somewhat more rapidly with acetone than with benzene as a solvent for 0.3 per cent methylcholanthrene. Mice painted with a 0.3 per cent solution of methylcholanthrene in acetone 3 times a week and with pure benzene on alternate days developed cancer even more rapidly. Fifty-six per cent of the 123 mice painted with methylcholanthrene in these experiments had multiple malignant neoplasms of the skin.

The hyperemia of the skin of a rabbit's ear after the application of methylcholanthrene in benzene was a combined effect of the carcinogen and of its solvent. The less pronounced hyperemia after the application of methylcholanthrene in acetone was caused by the carcinogen alone, since pure acetone did not produce appreciable hyperemia.

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Influence of Toxic Amounts of Estrin Upon Intact and Castrated Male Marsh-Buffalo Mice*

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Evidence (1, 2, 3, 4) has been produced indicating that the Marsh-Buffalo strain of mice is a stumbling block to any attempt to correlate tendency to development of cancer of the mammary gland with the amount of exogenous estrin required to enhance the incidence of this form of cancer in females or to produce it in males. Amounts of estrin which enhance formation of breast tumors in other high-cancer strains of mice fail to do so in either males or females of the Marsh-Buffalo strain. By the use of larger and toxic amounts of estrin in the female, an increased tendency to formation not only of breast tumors, but also of lymphoid tumors has been noted. The purpose of the present experiment was to ascertain whether these toxic doses could produce tumors of the mammary gland in males, whether the gonads were involved and to what extent a response, if elicited, was produced in tissues other than those of the mammary gland.

MATERIALS AND METHODS

Two treated groups of 36 mice each and one control group of 43 mice were segregated by random sampling at the age of 1 to 1½ months. At this time the mice of one treated group were castrated. Administration of the estrogen was begun 1 week later. The crystalline hormone (estradiol)[1] was dissolved in sesame oil in a concentration of 2 mgm. per cc. This was first administered parenterally in doses of 0.04 cc. per mouse twice weekly to both treated groups. The schedule of dosage, the amounts of hormone administered per mouse, and the periods of noninjection, which were interposed when toxic manifestations appeared, follow: 1.36 mgm. hormone per mouse in 60 days, 40-day rest period, 0.64 mgm. hormone in 30 days, 45-day rest period, 0.32 mgm. hormone in 10 days, 80-day rest period, 1.20 mgm. hormone in 95 days, at the end of which time dosage was discontinued.

In Table I are recorded, for the two treated and control groups, the cumulative percentage incidences of lymphoid tumors, breast tumors (adenocarcinoma), and death due to causes not related to tumor formation. The latter data also included mice sacrificed in a moribund condition. The experiment was ended when the mice had reached the age of 15 months as the numbers of surviving mice in the treated series were not large enough to produce additional data which would have significance when subjected to statistical analysis. At this time 12 mice were alive in the castrated group, 10 mice alive in the intact group, and 33 mice alive in the controls. Twenty-five per cent of the intact dosed mice developed scrotal hernias during the course of the experiment. One of the castrated mice and one control mouse developed a fibrosarcoma.

RESULTS

Toxicity.—It was originally planned to administer 3 to 4 mgm. of estrogen per mouse in a 5- to 6-month period as was done in the experiments with female Marsh-Buffalo mice, but the physical condition of the mice after a 2-month period did not warrant a course of continuous treatment. The mice received 2.3 mgm. estrogen per mouse in the first 6-month period and a total of 3.5 mgm. in a 12-month period, so that the total amount was approximately the same as that received by the females. The incidence of death due to causes not related to tumor formation in the series of treated intact mice demonstrates that the amount of estrogen administered was toxic. The high incidence of spontaneous death which occurred when the females received the massive dosage in a 6-month period was avoided. Both series of treated mice increased in weight during the course of the experiment. When first dosed the mean body weights were respectively 15.6 and 16.0 gm. At the age of 11 months the weights were 26.7 and 28.0 gm. respectively.

Tumors.—The incidence of lymphoid tumors for the intact treated male mice is not significantly above the range obtained for the controls. In contrast the high incidence for the castrated mice should be noted, the difference being significantly greater.

No adenocarcinomas of the breast occurred in any

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[1] The authors are indebted to Dr. Erwin Schwenk of the Schering Corporation for the supply of estradiol.

but the castrated group, in which only 2 tumors, not differing histologically from those found in females, appeared.

DISCUSSION

The control mice in the present experiment did not receive injections of sesame oil, which we (2) had previously found was slightly toxic to the female and decreased somewhat the incidence of tumors of the breast. Since the male mouse does not develop tumors of the breast spontaneously and since the incidence of lymphoid tumors in control and treated intact mice was the same, the influence of sesame oil in the present experiment cannot be measured.

In the female Marsh-Buffalo mouse, toxic doses of estrin increased the incidence of lymphoid tumors in both intact and ovariectomized animals, and the results of the present experiment are of particular interest in that the castrated male showed a significant increase in incidence of lymphoid tumors in contrast to the intact male. Since the comparison between intact and castrated mice for both sexes was made under strictly controlled experimental conditions (housing, food, injection of sesame oil), it may be concluded that the male but not the female gonad is involved in a defense mechanism. In the case of the adenocarcinoma of the breast the results are similar but less striking. In view of the fact that the occurrence of breast tumors in untreated male mice of the Marsh-Buffalo strain has not been observed in this laboratory or reported from other laboratories, the occurrence of 2 breast tumors in the castrated dosed mice would hardly be fortuitous. The appearance of only 2 tumors confirms previous deductions that the strain is highly resistant to the action of exogenous estrin; that when the action can be demonstrated, it is only after the administration of toxic amounts; that under such conditions the action is not confined to tissues of the mammary gland and the effect upon lymphoid tissue is more marked than the effect upon the breast.

Table 1: Cumulative Incidence of Lymphoid Tumors, Adenocarcinoma of Breast, and Death Due to Causes Not Related to Tumor Formation in Intact and Castrated Male Marsh-Buffalo Mice Which Had Received Estrogen and in Controls

	Dosed—castrated (36 mice)			Dosed—intact (36 mice)			Control (43 mice)		
Age in months	Per cent dead of other causes	Per cent lymphoid tumors	Per cent adeno-carcinoma	Per cent dead of other causes	Per cent lymphoid tumors	Per cent adeno-carcinoma	Per cent dead of other causes	Per cent lymphoid tumors	Per cent adeno-carcinoma
5	0	0	0	8	0	0	0	0	0
6	11	3	0	11	0	0	0	0	0
7	11	11	0	20	0	0	2	0	0
8	17	19	0	22	0	0	5	0	0
9	17	22	0	28	3	0	5	0	0
10	20	29	0	28	8	0	5	0	0
11	20	29	6	28	8	0	5	0	0
12	20	31	6	33	8	0	7	2	0
13	22	31	6	44	8	0	7	5	0
14	25	34	6	56	8	0	16	5	0

To date carcinoma of the breast has not been produced in intact male Marsh-Buffalo mice by the administration of estrogens over a long period of time either in toxic or nontoxic doses.

SUMMARY AND CONCLUSION

Estradiol in sesame oil was administered in a total dose of 3.5 mgm. per mouse intermittently over a 12-month period to groups of castrated and intact male Marsh-Buffalo mice.

In the castrated mice the incidence of lymphosarcoma was increased significantly above that occurring in both estrin-treated and control intact mice.

No mammary gland tumors appeared in the series of intact males and only 6 per cent (2 tumors) developed in the castrated group, which had received estrin.

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Quantitative Aspects of the Antifibromatogenic Action of Synthetic Desoxycorticosterone Acetate*†

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The results of the experiments of Lipschütz and Iglesias (3) and Iglesias (1) showing that abdominal fibroids can be induced in almost every castrated female guinea pig subjected to prolonged treatment with subcutaneous injections of estrogens offered the opportunity to study also the antitumoral action of certain gonadal steroids. Fibroids induced by estrogens were prevented by the simultaneous administration of progesterone (4) or testosterone (9). The most convenient method for such experiments is the implantation of tablets under the skin by the technic of Deanesly and Parkes. The fibromatogenic tablet of estradiol was implanted on one side of the body; a tablet of progesterone was implanted on the other side of the same animal (6). On account of the great similarity between progesterone and desoxycorticosterone as to chemical structure it was thought that this cortical hormone may also have antifibromatogenic activity. This has been proved to be true. In experiments with the simultaneous implantation of tablets of estradiol and desoxycorticosterone acetate (7, 8, 11), this synthetic cortical hormone was found to have great antifibromatogenic activity. Uterine bleeding induced by the estrogen was also prevented by desoxycorticosterone acetate.

When dealing with the comparative antifibromatogenic activities of steroids, quantitative results were expressed as a ratio between the fibromatogenic estrogen and the antifibromatogenic steroid. This ratio was more favorable with desoxycorticosterone acetate than with testosterone propionate (8). The quantity of desoxycorticosterone able to prevent fibroids was only about three times that of the fibromatogenic α-estradiol. But the question arises whether the preventive effect really depends upon a given ratio between the fibromatogenic and the antifibromatogenic steroid, or whether the antifibromatogenic action is governed by other quantitative laws. These quantitative aspects are also of fundamental importance for the whole problem of the antagonism of sex hormones, as was recognized years ago in work with ovarian grafts by Lipschütz and his associates (10).

EXPERIMENTS

Fragments of tablets of α-estradiol and synthetic desoxycorticosterone acetate were implanted under the skin of 42 castrated female guinea pigs. The tablets were prepared by compression. The weight of the fragments used varied greatly. By this means variable ratios between the quantities of absorbed estradiol and absorbed desoxycorticosterone were obtained. The animals were sacrificed 57 to 64 days later. A group of 8 animals into which only tablets of estradiol were implanted served as controls. The results are given in Table I and examples are pictured in Figs. 1-3.

Uterine and extragenital fibroids were produced with as little as 1.8 to 3.3 mgm. of estradiol absorbed in the course of about 2 months. The average tumoral effect was more than class 5 in the control group (Table I, group A) according to our classification (2, 5). It must be emphasized that the result with control animals is not always such a remarkable one; an average of only 3 is sometimes obtained under similar experimental conditions (7).

Uterine fibroids were absent and the extragenital fibrous reaction was markedly reduced when 6 to 8 mgm. of desoxycorticosterone were absorbed simultaneously with the estrogen (Table I, group B). The average tumoral effect was 1 in this group; this average was due to small nodules on the spleen (tumoral seed) and to fibrous peritoneal strands, a reaction which is characteristic of a diminished sensibility for the tumorigenic action of estrogens as shown by Vargas and Lipschütz (12). There was not a single animal with an individual spherical tumor of class 1 (about 1 mm. in diameter).

The preventive action of similar quantities of desoxycorticosterone is still observable when the quantity

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of the fibromatogenic estradiol is considerably raised (Table I, group C). The ratio between the absorbed estradiol and the absorbed desoxycorticosterone dropped in group C to 1:1.3, instead of 1:3 in group B; diminution of the ratio had consequently no influence on the result.

In group D the same quantity of estrogen was absorbed as in group B but the quantity of desoxycorticosterone was diminished below a total of 6 mgm. absorbed in the course of 2 months. The average ratio between estradiol and the cortical steroid in group D was no less than in group C. Notwithstanding that 10 out of 20 animals had individual uterine or extragenital tumors of class 1, 3 animals had individual tumors intermediate between 0.5 and 1. The average tumoral effect in group D was considerably greater than in groups B and C. Uterine fibroids, especially extragenital ones, were observable in group D even when the ratio between estradiol and desoxycorticosterone was as high as 1:2.

TABLE I: FIFTY CASTRATED FEMALE GUINEA PIGS (325 TO 610 GM.) WITH SUBCUTANEOUS TABLETS OF ESTRADIOL AND DESOXYCORTICOSTERONE ACETATE

Series XXXI and XXXII, group	Number of animals	Initial weight of tablets		Quantity absorbed		Ratio of steroids	Tumoral effect[4]	Animals with individual tumors of class 1[5].
		Estradiol, mgm.	Desoxycorticosterone acetate, mgm.	Estradiol, mgm.	Desoxycorticosterone,[3] mgm.			
A	8	7.2 (3.4–9.6)[2]	.....	2.2 (1.8–3.3)	0	0	5.7 (1.5–2.5–3–3–7.5–9–9–10)	7
B	8	7.6 (3.7–11.2)	8.7 (7.2–10.0)	2.3 (1.6–3.1)	6.8 (6.1–7.9)	1:3 (2.3–4.1)	1.1 (0.5–1.5)	0
C[1]	14	24.2 (13.4–38.5)	19.9 (16.2–38.5)	7.8 (5.5–13.5)	10.0 (6.8–14.6)	1:1.3 (0.8–1.6)	0.5 (0–1.5)	0
D	20	7.2 (3.0–10.0)	4.4 (1.3–8.2)	2.0 (1.3–3.0)	3.1 (1.2–5.1)	1:1.6 (0.6–3.3)	2.5 (11 animals: 1, 2, 2.5, 2.5, 3, 3.5, 3.5, 3.5, 4.5, 5, 6.5)	10(3)[6]

[1] Belonging to a series of Nuñez (11).
[2] Figures in parentheses give range.
[3] Figures calculated for the *free* steroid.
[4] According to our system of classification (2, 5).
[5] Spherical tumors of 1 mm. in diameter.
[6] Figure in parentheses indicates tumors transitional between 0.5 and 1.

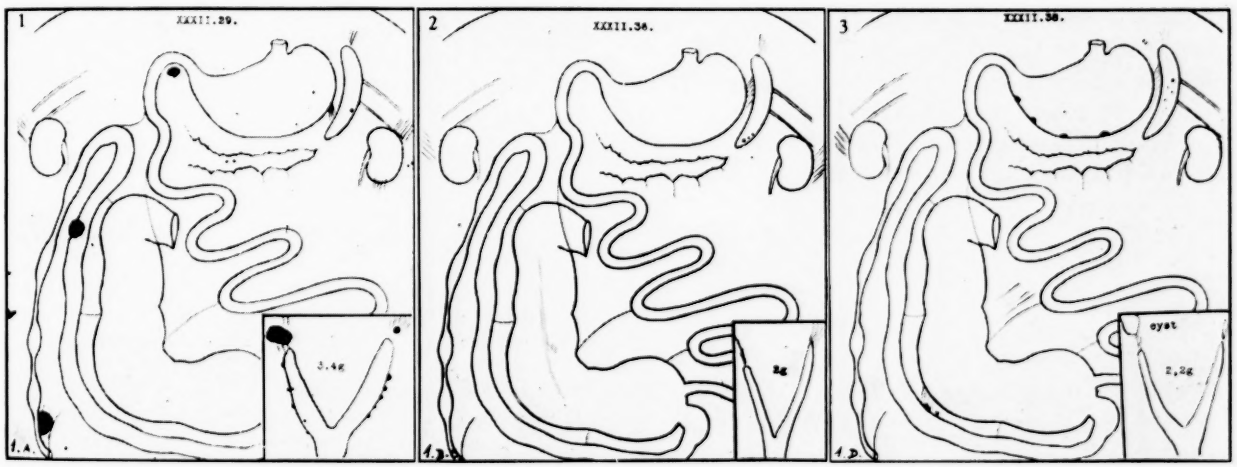

FIG. 1.—Diagram of uterine and extragenital tumors in animals of group A. Absorption of 1.9 mgm. estradiol in 56 days. Total tumoral effect, 7.5 (above average). Series XXXII. 29.

FIG. 2.—Diagram of tumoral effects in animals of groups B and C. Absorption of 2.6 mgm. estradiol and 6.8 mgm. desoxycorticosterone in 57 days. Ratio, 1:2.6. Maximal reaction typical for these groups. Total tumoral effect, 1.5. Series XXXII. 36.

FIG. 3.—Diagram of tumoral effects, apical uterine and extragenital tumors, in animals of group D. Absorption of 1.9 mgm. estradiol and 4.6 mgm. of desoxycorticosterone (from a pellet of 7.6 mgm. of the acetate) in 57 days. Ratio, 1:2.4. Total tumoral effect, 3.5. Series XXXII. 38.

DISCUSSION

When comparing the results in group D with those in group C (Table I) one may conclude that the same ratio which at higher levels of the fibromatogenic and antifibromatogenic steroids prevents fibroids (group C) may lose this preventive action at lower levels of these steroids (group D). But the following objection can be raised against this conclusion. In experiments in which sufficiently small quantities of desoxycorticosterone acetate were implanted (Table I, group D), absorption proceeded very far in the course of 2 months; in only 8 out of 20 animals were 1.3 to 2.5 mgm. of desoxycorticosterone acetate recovered at necropsy. In the remaining 12 animals of this group absorption was complete or almost complete, the quantities recovered at necropsy being nil or no more than 1 mgm. One might then assume that tumors developed in group D because the pellets had diminished appreciably long before the animals were sacrificed and that for a time sufficient for production of tumors the ratio was below that which was active in group C. This possibility cannot be denied. But this would apply only to a restricted number of cases because only 7 out of the 12 animals mentioned with complete or almost complete exhaustion of the antifibromatogenic steroid corresponded with those animals of group D which had fibroids of class 1 (Table I, last column).

A summary of our results is presented in Fig. 4 in which the quantities of desoxycorticosterone absorbed per day in the above 42 experiments (groups B to D) are given as ordinates against the absorbed quantities of estradiol on the abscissa. The figures of absorption *per day,* however, are not quite exact; absorption per day diminishes with time when the surface of the pellet has diminished, as shown by different workers. This refers to both steroids, fibromatogenic and antifibromatogenic. Also, as already pointed out, in a number of experiments with tumors the objection is valid that absorption was completed or nearly completed before necropsy (see dot beneath the signs in Fig. 4).

In Fig. 4, the straight diagonal line *ab* indicates quantities of desoxycorticosterone equimolecular with those of estradiol (mol. wt. of estradiol, 272; mol. wt. of desoxycorticosterone, 324). Fig. 4 would allow for the assumption that the fibromatogenic action of the estrogen can be inhibited by an equimolecular quantity of desoxycorticosterone but that this equimolecular ratio is antitumorigenic only at high levels of the estrogen because the inhibitory effect of desoxycorticosterone would depend also upon a threshold dosage of this steroid. The threshold dosage would be in our experiments about 90 μgm. per day (the horizontal line a_1b_1 in Fig. 4). This corresponds to about 0.2 mgm. of desoxycorticosterone per kg. body weight per day. Approaching this quantity the source, if it is a very small pellet, may become exhausted or almost exhausted prematurely. This invalidates somewhat our statements referring to the threshold dosage.

The danger of dropping below a threshold should be considerable when one tries to maintain it with daily injections instead of using pellets. This may serve as a hint in clinical application.

One may question whether Fig. 4 would allow also for the assumption that the antifibromatogenic effect depends solely upon a given threshold dosage of the antifibromatogenic steroid irrespective of the ratio's being equimolecular or not. Subsequent experiments must decide whether such an assumption would be justified.

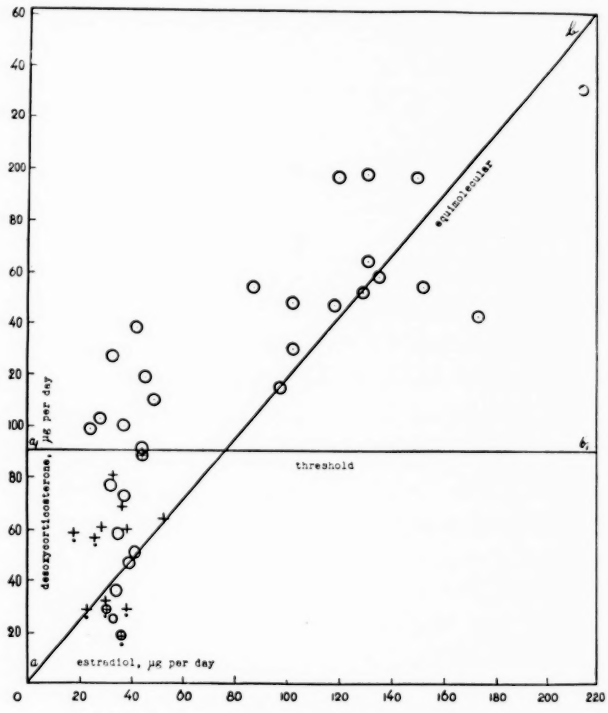

FIG. 4.—Tumoral reaction in 42 female guinea pigs with subcutaneously implanted tablets of both estradiol and desoxycorticosterone acetate. Absorption of varying quantities of both hormones.

○ = animals without fibroids.

\+ = animals with fibroids.

⊕ = animals with individual fibroids transitional between 0.5 and 1 (see group D).

The dot . below + or ⊕ signs indicates recovery of no more than 1.0 mgm. of desoxycorticosterone acetate at necropsy. This is not indicated for the remaining 7 animals denoted by the sign ○.

SUMMARY AND CONCLUSIONS

When tablets or pellets of estradiol and synthetic desoxycorticosterone acetate are implanted simultaneously under the skin of the female guinea pig, the

fibromatogenic action of the estrogen can be inhibited by equimolecular quantities of the adrenal cortical steroid. This holds only for sufficiently high levels of the fibromatogenic estrogen; at lower levels the equimolecular ratio often loses its inhibitory action and the quantity of the antifibromatogenic steroid necessary to prevent fibroids is then a multiple of the tumorigenic one. This outcome may be explained by the assumption that the inhibitory effect depends also upon a threshold dosage of the antitumorigenic steroid. Ineffectiveness of very small pellets may depend also on a premature more or less complete absorption of the source of the antifibromatogenic steroid.

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The Relation of the Antifibromatogenic Activity of Certain Steroids to Their Molecular Structure and to Various Actions of These Hormones*†‡

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Three different synthetic steroids, progesterone, desoxycorticosterone acetate, and testosterone propionate, have been shown to have the capacity of preventing abdominal fibroids elicited by estrogens. The question arises as to which are the special molecular structures responsible for the antifibromatogenic activity of these compounds. Different structures are of interest here (Fig. 1): first, the ketonic oxygen in position 3 and the double bond, △ 4-5, in the ring I which are common to the three steroids; second, the short side chain of carbons 20 and 21 attached in position 17 by which progesterone and desoxycorticosterone differ from testosterone. The importance of these molecular conditions will be discussed by comparing the antifibromatogenic activity of these three steroids and of a fourth, androstanediol (Fig. 1, IV), in which the double bond and the ketonic oxygen in position 3 are lacking.

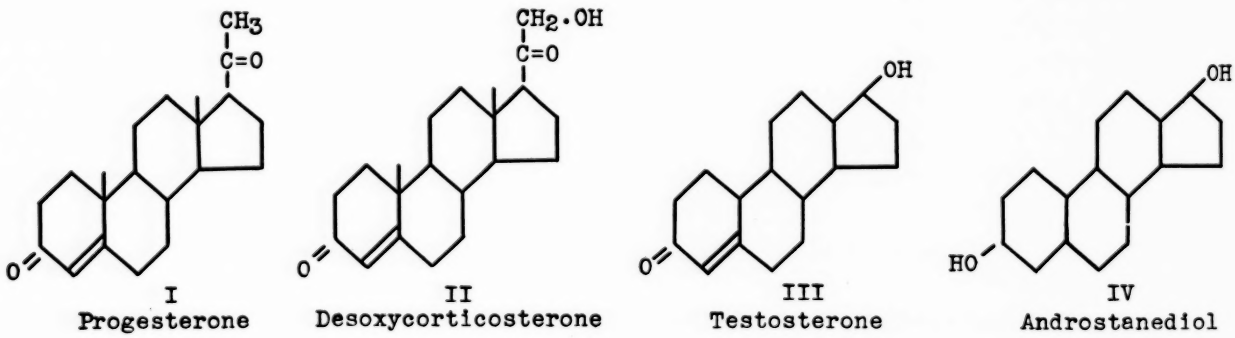

FIG. 1

EXPERIMENTAL

A total of 173 animals was used for comparison (Tables I and II). The control group was of animals treated with subcutaneous tablets of estradiol only; other groups were of animals in which tablets of estradiol and of one of the above-mentioned four steroids were simultaneously present. The 27 animals of the control group (a) of Table II were chosen from different experimental series; only animals in which absorption of estradiol per day was not greater than in the remaining groups were used. On account of the great variation one meets in this kind of experiment, a second series of 20 animals, group (b) in Table II, was included in the control group. This series (b) was chosen because it has given so far a lower *average* tumoral effect with tablets of estradiol than any found in similar control groups; for details of series (b) see Table I of the thesis of Nuñez (17). The desoxycorticosterone group is the same as that of the preceding paper (13). As to the progesterone, testosterone, and androstanediol groups, greater details will be found in the thesis of González and Vera (in preparation). The animals of the testosterone-treated group were also cited partly from previous work by Lipschütz, Vargas, and Nuñez (16). The weight of the tablets, or fragments, of the fibromatogenic estradiol and the antifibromatogenic steroids used in the 173 animals of Tables I and II was between 0.6 and 90 mgm. In this way the necessary variation of quantities absorbed was assured, since absorption depends on the surface.

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‡ The senior author is responsible for the interpretation of results and the preparation of the manuscript.

Comparison was made on the basis of the following four criteria:

1. The daily threshold quantity of the antifibromatogenic steroid, or the minimum quantity above which individual spherical fibroids of class 1 (about 1 mm. in diameter) were absent. The fibrous reaction consisted in these animals of small nodules only, "tumoral seed," or fibrous strands. This type of extragenital reaction was always present in our work with antifibromatogenic steroids. Indeed we are as yet not sure whether the minimum quantity is likely to give an exact expression of the antifibromatogenic faculty of the steroid. But, as explained in the preceding paper (13), neither can the ratio between the fibromatogenic and antifibromatogenic steroids we have as yet used for this purpose (15-17) be considered as an exact measure in this kind of experiment.

2. The average tumoral effect in the respective group. We have emphasized that the tumoral effect is very variable. The standard deviation of the average and the significant difference also have been calculated according to the method of Burn (1).

3. The number of animals which reached in the respective group the average tumoral effect of the control group.

4. The number of animals which had individual tumors of class 1 (see above).

The results are summarized in Table II. Details referring to experiments with progesterone, testosterone, and androstanediol are to be found also in Fig. 2 of the present paper; for details referring to desoxycorticosterone see Fig. 2 of the previous paper (13).

As seen from Table II, androstanediol behaved differently from the other 3 steroids: abdominal fibroids were produced even when 224 μgm. of androstanediol daily were given against 77 μgm. of estradiol, or 167 μgm. against 40 μgm. The average tumoral effect in the androstanediol group was higher than in the control series (b); the difference between the androstanediol group and the control series (a) was not significant. Neither was there any difference between the androstanediol and control group as to the number of animals which reached the average tumoral effect of the control series especially when compared with the series (b), or as to the number of animals with tumors class 1. It is evident that there was no inhibitory effect with androstanediol under the present experimental conditions. This does not exclude the possibility that greater quantities of this steroid

TABLE I: WEIGHT OF TABLETS AND QUANTITIES ABSORBED IN THE DIFFERENT GROUPS

Group	Tablets of estradiol: Initial weight, mgm.	Tablets of estradiol: Absorbed, mgm.	Tablets of antifibromatogenic steroid: Initial weight (esterified steroid), mgm.	Tablets of antifibromatogenic steroid: Absorbed (free steroid), mgm.
Control	0.6-24.0	0.3- 4.1	.....	.....
Progesterone	8.4-28.1	1.2- 4.5	6.8-35.0	3.4-13.6
Desoxycorticosterone acetate	3.0-38.5	1.3-13.5	1.3-38.5	1.2-14.6 *
Testosterone propionate	3.2-27.0	1.1- 4.2	10.8-32.6	4.5-14.0 *
Androstanediol propionate	9.0-22.0	1.9- 4.3	10.5-89.8	1.5-12.5 *

* Values obtained by dividing the original figures by 1.13, 1.19, and 1.19 respectively.

TABLE II: SUMMARY OF RESULTS

Substances and quantities absorbed per day, μgm.*	Anti-fibromatogenic threshold, μgm.	Average total tumoral effect, (T.T.E.)	Significant difference ‖	Number of animals: Total	Number of animals: With tumors, class 1 †	Number of animals: Reached average T.T.E. of control group	Duration, in days
Estradiol-control 5-63 (a)	.....	4.9 ± 0.56 §	..	27	22 (2)	12	50-62
[35-74] (b)	.....	[3.0 ± 0.54]	..	[20]	[14]	[10]	[54-62]
Estradiol 19-62 and progesterone 54-220	± 65	1.3 ± 0.07	6.3 [3.1]	28	0 (4)	0 [0]	62-64
Estradiol 18-214 and desoxycorticosterone acetate 20-231	< 90	1.6 ± 0.22	5.5 [2.4]	42	10 (3)	2 [7]	57-64 ‡
Estradiol 17-75 and testosterone propionate 70-225	200	1.4 ± 0.35	5.3 [2.5]	25	7	3 [4]	55-65
Estradiol 34-77 and androstanediol propionate 26-224	> 224	3.5 ± 0.59	1.7 [ ] ¶	11	8	3 [8]	55-57

* Quantities of free hormones are given.
† Figures in parentheses: animals intermediate between 0.5 and 1.
‡ Four animals 70 to 73 days.
§ $\epsilon = \sqrt{\frac{\Sigma d^2}{n(n-1)}}$ (standard deviation of the average).
‖ $\frac{m_{ctr} - m_{exp.}}{\sqrt{\epsilon^2_{ctr} + \epsilon^2_{exp.}}}$ (significant difference); m = average.
¶ $m_{exp.}$ greater than $m_{ctr.}$

may be active. But our results show that the antifibromatogenic activity is reinforced by the double bond in ring I or the ketonic oxygen in position 3 (see especially Fig. 1, III and IV). It cannot be said whether reinforcement of antifibromatogenic activity is effected through the simultaneous presence of both these structural conditions or whether only one of both is relevant. This question is under investigation.[1]

Testosterone has considerable antifibromatogenic activity. The difference between the average tumoral effect in the testosterone and the control groups is significant; the number of animals in the testosterone group reaching the average of the control groups is small. Is there also a difference between the testosterone-treated group and those treated with progesterone and desoxycorticosterone? The determination of the antifibromatogenic threshold was certainly not an exact one. But the distance between the threshold values for testosterone on one side, and for progesterone and desoxycorticosterone on the other, was considerable. As to the other criteria, the difference between testosterone and progesterone also was remarkable. All this points to a lesser antifibromatogenic activity of testosterone as compared with that of progesterone. One may suppose that the difference was due to the absence of the short lateral chain of carbons 20 and 21 (Fig. 1, I and III).

According to our results the antifibromatogenic activity of progesterone was apparently also greater than that of desoxycorticosterone acetate. This would raise the question of whether oxidation in position 21 by which desoxycorticosterone differs from progesterone (Fig. 1, I and II) attenuates the antifibromatogenic activity. At present we do not feel warranted in expressing a definite opinion on this subject on account of the difficulties of the exact determination of the threshold quantity (13).

DISCUSSION

Notwithstanding the difficulties which present themselves when one tries to interpret our quantitative results with the antifibromatogenic action of different steroid hormones, three fundamental observations attract attention: first, steroid hormones prevent fibroids elicited by estrogens; second, preventive activity of steroids differs quantitatively and these differences coincide with those of molecular structure; third, a cortical hormone competes quantitatively with progesterone as a powerful antifibromatogenic agent. The descending order of antifibromatogenic activity is most likely the following: progesterone > desoxycorticosterone > testosterone > androstanediol. Since it was in experiments with estrogens that the antifibromatogenic action of certain steroids and their descending order was studied one must raise the question whether this inhibitory action is coincident with an antiestrogenic action in general.

Antifibromatogenic and antiestrogenic activity.— Antifibromatogenic action of steroids in our experiments was certainly concomitant with an inhibitory action against several other effects due to estrogens, such as the increase of the uterine weight, uterine bleeding, glandular hyperplasia, and formation of polyps which also were partly counteracted (unpublished results). Development of the mammary gland was not diminished. But the most essential question is whether antifibromatogenic and antiestrogenic effects follow the same descending order; *i.e.*, whether greater antiestrogenic activity is coincident with greater antifibromatogenic action. So far this can be discussed quantitatively only for the following antiestrogenic actions: 1. inhibition of the cornifying action of the vaginal mucosa of the rat and mouse, and 2. inhibition of the increase of uterine weight and of the opening of the vagina in the guinea pig. According to Courrier and Cohen-Solal (2, 3) and to Robson (18) the quantities of progesterone which were necessary to inhibit the cornifying action of estrogens (estrone, estradiol) on the vaginal mucosa of the rat or mouse were greater than those of testosterone. On the contrary, in our work with guinea pigs, greater antifibromatogenic activity of progesterone and desoxycorticosterone when compared with testosterone was coincident with a more effective inhibition of the increase of uterine weight and of the opening of the vagina as shown by Lipschütz, Vargas, and Nuñez (16), and likewise with a more pronounced inhibiting action against atypical proliferation of the uterine epithelium (unpublished results).

Androstanediol inhibited cornification of the vaginal mucosa in the mouse, according to Robson (18); it was many times less active than testosterone but superior to progesterone. In our experiments with guinea pigs androstanediol, whose antifibromatogenic activity was nil, had neither an inhibiting action against the increase of the uterine weight nor against the opening of the vagina.

The descending order of antiestrogenic activity in the rat and mouse is not identical with that in the guinea pig. This gives evidence that our statements made for one species with reference to the coincidence of antifibromatogenic and antiestrogenic activities are not valid for other species. When making the attempt to bring both types of action into a quantitative agreement one should also not forget that various steroids

[1] In the meantime we have found that the double bond in ring I is not essential for antifibromatogenic activity when the ketonic oxygen in position 3 is present. Dihydrotestosterone (propionate) in which the double bond is lacking was still antifibromatogenic, contrary to androstanediol (propionate) in which both the double bond and the ketonic oxygen are absent.

such as testosterone and androstanediol have not only an antiestrogenic, but also a "cooperative" action, in the sense that they augment the stimulating action of estrogens on the genital tract (9). An estrogenic action has been demonstrated by Speert (20) even for so as our own work is concerned there is evidence that this is not the case. A transformation of the clitoris into a hypospadic penis-like organ identical with that which has been observed under the influence of a testicular graft (11, 12) was effected with quantities

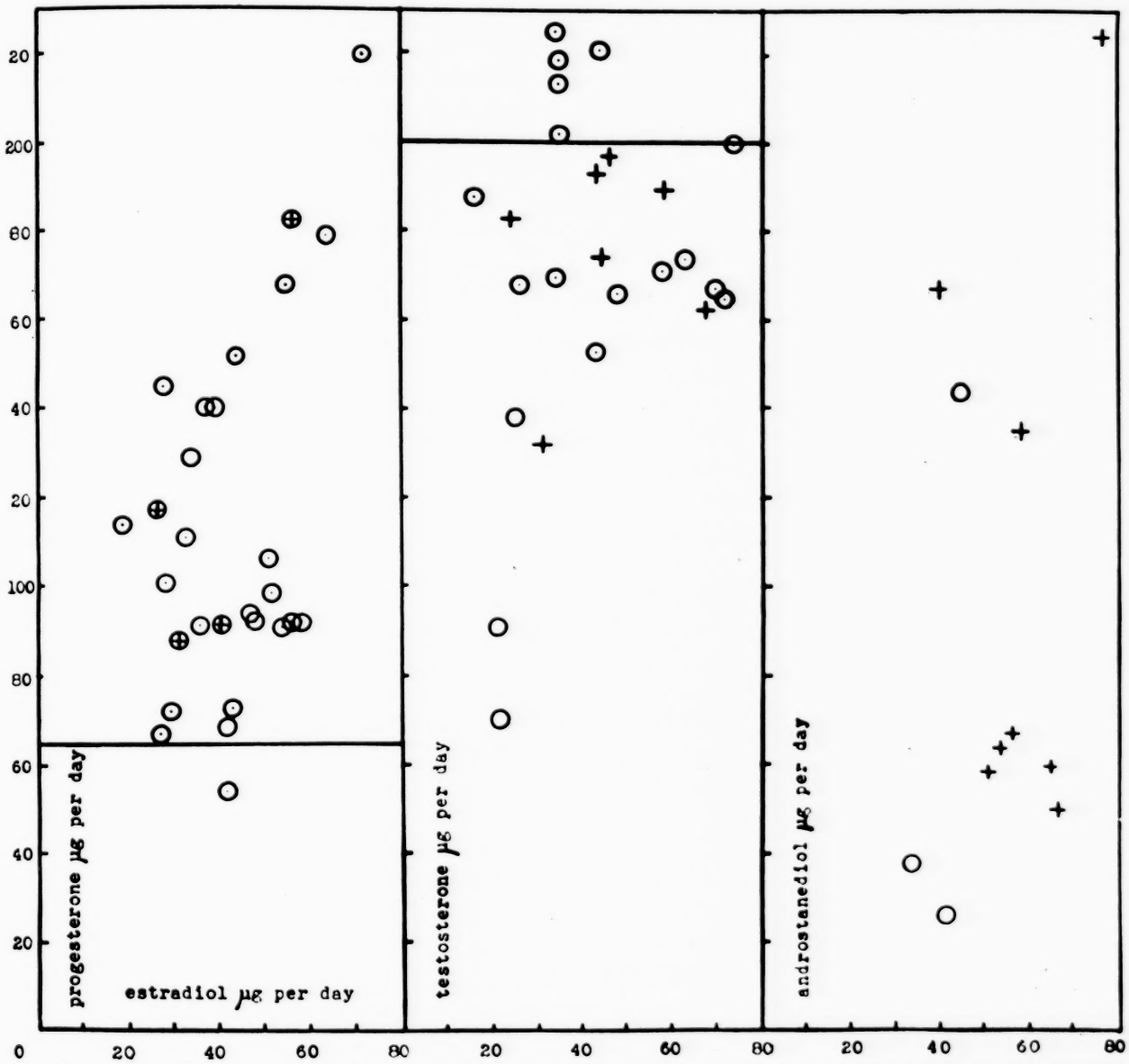

FIG. 2.—Graphical summary of data from Tables I and II showing the tumoral reactions in female guinea pigs with subcutaneously implanted tablets of both estradiol and antifibromatogenic steroids. Varying quantities of the hormones were absorbed.

○ = animals without fibroids.
+ = animals with fibroids.
⊕ = animals with individual fibroids transitional between classes 0.5 and 1.

powerful an antifibromatogenic steroid as desoxycorticosterone.

Antifibromatogenic and masculinizing activity.—One must also question whether the descending order of the antifibromatogenic activity of steroids is coincident with that of their masculinizing activity. As far of testosterone, antifibromatogenic or not (14, 19). This masculinizing effect was never observed with dosages of progesterone or desozycorticosterone much greater than those necessary for antagonizing the fibromatogenic effect of the estrogen (14, 15). It is well known that these steroids can exert a masculinizing

action; but our results show that the antifibromatogenic threshold is certainly smaller than the masculinizing one with these two steroids.

As to androstanediol, the quantities used which were not antifibromatogenic did not produce the masculinizing effect on the clitoris.

Antifibromatogenic and progestational activity.— The three antifibromatogenic steroid compounds have also a progestational action. Comparative data as to the progestational activity of the three compounds are not very numerous; but it decreases apparently from progesterone to desoxycorticosterone and testosterone according to Klein and Parkes (8) and Leathem and Crafts (10). No data are to be found in the literature on any progestational activity of androstanediol, as indicated by Korenchevsky (9). Though all these quantitative data and likewise those referring to antifibromatogenic activity are by no means exact there is much probability that the descending order of both these activities of progesterone, desoxycorticosterone, and testosterone is the same.

This discussion is summarized in Table III.

Table III: Comparative Activities of Steroids

Action	Descending order of activity	Authorities
Antifibromatogenic	Progesterone > desoxycorticosterone > testosterone > androstanediol	The present paper
Antiestrogenic (inhibition of increase of uterine weight and of opening of vagina, in guinea pig)	Progesterone and desoxycorticosterone > testosterone > androstanediol	Lipschütz, Vargas and Nuñez (16) Vera (unpublished)
Antiestrogenic (vaginal mucosa of the castrated rat)	Testosterone > androstanediol > progesterone	Courrier and Cohen-Solal (2, 3) Robson (18)
Masculinizing (hypertrophy of the clitoris of the guinea pig)	Testosterone > desoxycorticosterone and progesterone	Lipschütz and Vargas (13, 14) Ruz (16) Vera (unpublished)
Progestational (in the sensitized rabbit)	Progesterone > desoxycorticosterone > testosterone	Klein and Parkes (8) Leathem and Crafts (10)

It seems contradictory that the descending order of the antifibromatogenic and antiestrogenic activities of certain steroids should not coincide with that of masculinizing activities,[2] whereas the descending order of two activities so contradictory as the progestational and the antifibromatogenic should be the same. Progestational activity is associated with stimulation of proliferation of epithelial and conjunctive cells whereas antifibromatogenic activity means inhibition of proliferation of those cells which give origin to fibroblasts and connective fibers; and it will be shown in subsequent publications that antifibromatogenic action of progesterone and desoxycorticosterone is concomitant also with inhibition of epithelial proliferation. But one must emphasize that both stimulation and inhibition of proliferation may depend upon different quantitative levels of the antagonistic substances in play, as has been recently shown by Crabtree (4) in his work with the administration of different concentrations of chloracetones to the skin of animals painted simultaneously with carcinogenic hydrocarbons.

Our statement that antifibromatogenic activity coincides with the progestational effect is in accord with the findings of Klein and Parkes (8) who established that methylation or ethylation in position 17 reinforces the progesterone-like activity of testosterone and confers progesterone-like activity on androstanediol. Similarly, antifibromatogenic activity, according to our findings, is reinforced by the short side chain of carbons attached in position 17.

Our results are in accord also with clinical observation. It has been claimed for a long time that uterine fibroids are more frequent in virgin women than in nonvirgins; and that fibroids are more frequent in childless women as compared with women who have given birth to children (5). The assertion has been made also by Pinaud, quoted from Forgue and Massabuau (6), that their frequency was greater in women who became pregnant only at a more advanced age and had few pregnancies. One may tentatively assume that the longer action of progesterone exerted a preventive influence in women with pregnancies or with a greater number of pregnancies. Intervention of cortical steroids also has to be given attention in consideration of the causes of fibroids in women since it has been shown that desoxycorticosterone is a powerful antifibromatogenic factor.

Two parallel statements have been discussed above: first, that progesterone is seemingly the ideal physiological antifibromatogenic steroid, and second, that the descending order of the antifibromatogenic activity of a group of steroids was coincident with that of their

[2] But one should not forget that the short side chain attached in position 17 which reinforces progestational and antifibromatogenic activity diminishes masculinizing activity. This becomes especally clear when one compares the potency of testosterone and ethinyl-testosterone: addition of an ethinyl group to testosterone reduces its masculinizing activity about 600 times, as shown by Emmens and Parkes (6).

progestational activity. But it must be kept in mind that the second statement cannot be generalized. The possibility should not be excluded that when a greater number of steroids are examined, antifibromatogenic and progestational activities may be found to diverge and that a steroid may be eventually encountered which could be antifibromatogenic without having progestational activity.

SUMMARY

The faculty of preventing fibroids elicited by estrogens has been studied comparatively for different synthetic steroids with the purpose of relating antifibromatogenic activity to molecular structure.

The antifibromatogenic threshold, or the minimum quantity which must be released per day by a subcutaneous tablet of the antifibromatogenic steroid so as to inhibit fibroids, was lowest with progesterone; desoxycorticosterone (acetate) was next in order; testosterone (propionate) was highly active but less than progesterone and desoxycorticosterone; androstanediol (propionate) had no antifibromatogenic action.

The diminution of the average tumorigenic effect and other quantitative criteria of antifibromatogenic action were in accord with the statements referring to the antifibromatogenic threshold.

The question is discussed whether the descending order of the antifibromatogenic faculty of different steroids is coincident with that of their antiestrogenic and masculinizing activities. This was the case for the antiestrogenic activity in the guinea pig but not in the rat, and not for the masculinizing activity. There was a coincidence between the descending order of the antifibromatogenic activity as studied in the guinea pig and the progestational activity as studied in the rabbit. It would be unwise to generalize these statements on account of differences in the species.

Antifibromatogenic activity of steroids is apparently reinforced by the ketonic oxygen in position 3 or by the double bond, △ 4-5, ring I, or by both of these, and by carbons attached in position 17 (the short side chain of carbons 20 and 21). The latter is in accord with former statements of Klein and Parkes referring to the molecular conditions of progestational activity of steroids.

Thanks are due to Dr. L. Vargas, Jr., for help given in work with different antifibromatogenic steroids, and to Drs. C. Nuñez and J. Zañartu for help in experiments with desoxycorticosterone.

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On the Occurrence of Crystalline Material in the Lungs of Normal and Cancerous Swiss Mice

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The present investigation was undertaken with a view to studying histologically and cytologically the lungs of mice of a strain whose lineage is well known and which have proved remarkably susceptible to tumors of the lung. In initial studies of fixed and sectioned lung tissue, attention was at once attracted by bodies of an unusual sort which did not correspond to any known tissue or cell component. Further examination showed that these bodies were crystalline in nature.

Similar bodies had been noted by others who have studied lung tumors in mice. They were observed by Haaland (5) and also by Tyzzer (12). Neither of these workers knew anything of their origin. Haaland apparently did not recognize their crystalline nature but described them clearly as they appear upon first observation. Tyzzer, on the other hand, pictured and referred to them as crystals. Since more recent workers have made no mention of these bodies, it seemed desirable to make a more detailed study of their nature and incidence.

MATERIALS, METHODS, AND RESULTS

The mice used, supplied to the author through the kindness of Dr. Clara J. Lynch of the Rockefeller Institute for Medical Research, are descendants of the original so-called "Swiss" mice first brought to this country by Lynch in 1926. Starting in 1934 from 2 males and 4 females of the 10th generation of inbreeding, the mice in the present work represent all of 10 further generations bred in this laboratory. Slides or preserved material were also available from mice of several other strains.

Description of the Crystals

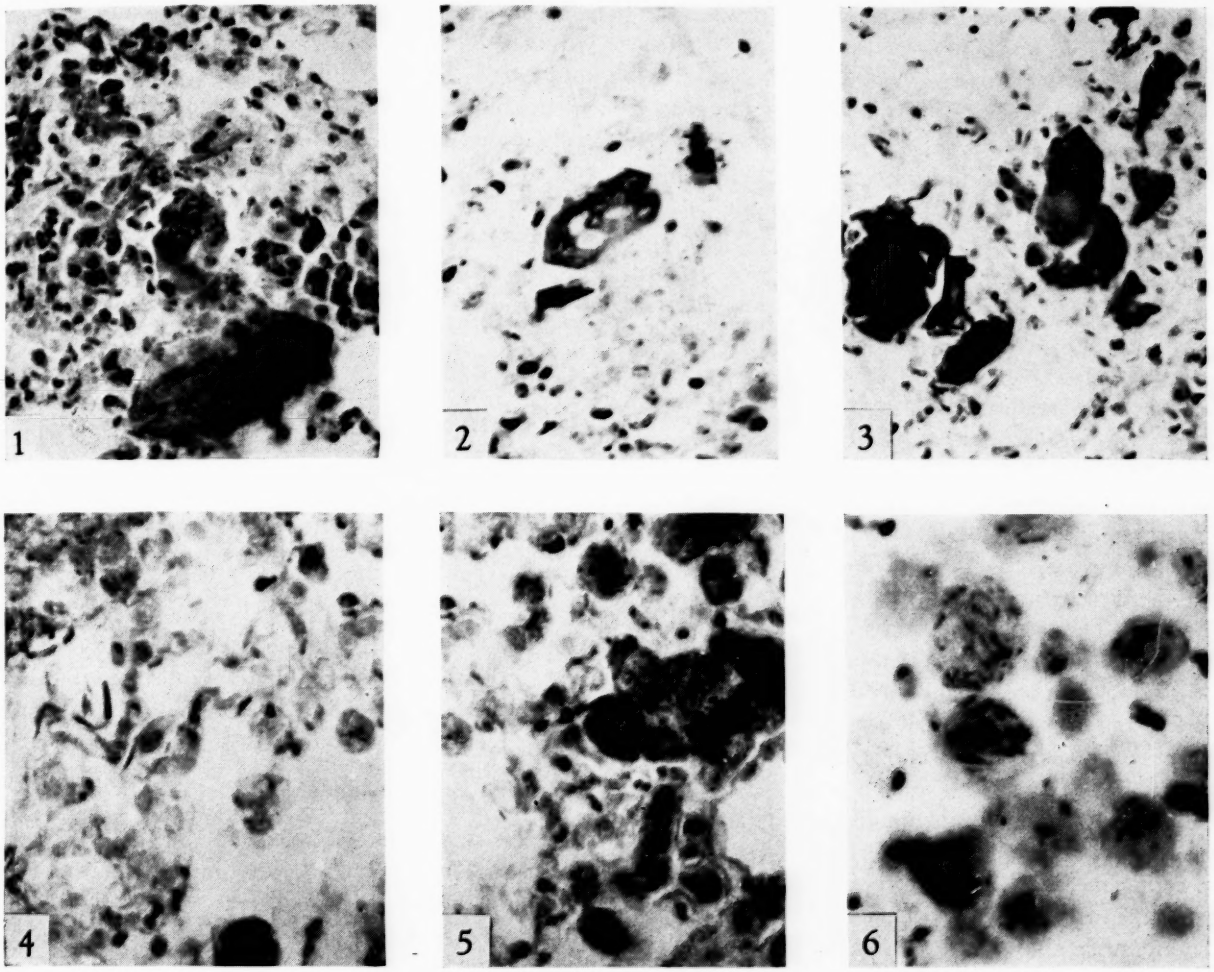
Form and location.—Large extracellular crystal plates are found most frequently in the lumina of the bronchioles. Less commonly they are lodged within the walls of the alveoli. In each case they are accompanied by masses of dust cells. The plates are six-sided, with two sides longer than the other four (Fig. 1). The striations within the plates appear after fixation and possibly represent cleavage planes along the surfaces of separate components from which the crystal has been built up. Frequently the plates show step-like indentations which may comprise all or part of the thickness of the crystal (Fig. 2). Crystals are found lying at all possible angles to the plane of section (Fig. 3). Typically they are about 3 μ thick, 35 μ broad, and 70 μ from point to point. Fig. 7 shows a series of camera lucida drawings of crystals upon which measurements of the angles were made. Crystals A and B, Fig. 7, are those shown in Fig. 1 and Fig. 2 respectively. The sums of the values of the complementary angles (a+c, a+d, b+c, and b+d) were found to be close to 180°, from which it follows that the opposed sides are parallel. Therefore it seems safe to assume that the plates are truly crystalline in nature.

Of far more common occurrence than the large plates are small crystals within the cytoplasm of the alveolar phagocytes or "dust cells." Fig. 6 shows a group of such cells lying free in an alveolar space, while Fig. 5 shows an aggregate of cells within the wall of an alveolus. The quantity of crystals in a cell varies from a single one to large numbers such as are shown in Fig. 5, where the cytoplasm is completely loaded. Dust cells with crystals show no differences from those which do not contain them other than hypertrophy of the cytoplasm and the frequent presence of two or more nuclei.

Crystals were seen in organs other than the lung in only three isolated instances: in the acinar cells of the pancreas; in the lumina of the glands of the endometrium of a mouse with a tumor of the uterus; in the tissue of a sarcoma of the thigh which involved the bone. Whether or not these unusual types of crystal are in any way related to the lung crystals cannot be stated at present.

Reactions to fixation and staining.—In fresh preparations of cells taken immediately from the lungs the crystals appear greenish and translucent. They do not stain supravitally with Janus green, neutral red, or eosin. They persist after all the common fixa-

* Presented to the Faculty of Bryn Mawr College in partial fulfillment of the requirements for the degree of Doctor of Philosophy.



FIGS. 1 TO 6 ARE PHOTOMICROGRAPHS OF SECTIONS OF THE LUNGS OF SWISS MICE.

FIG. 1.—Large extracellular crystal embedded in the wall of an alveolus; fragments of other plates may be seen among the neighboring dust cells. Mag. × 330.

FIG. 2.—Large extracellular crystal showing step-like indentations at one end; fragment of another plate nearby. Mag. × 330.

FIG. 3.—Large group of extracellular crystals. Mag. × 330.

FIG. 4.—Fragments of crystal plates of intermediate size lying free in the alveolar spaces. Mag. × 580.

FIG. 5.—Aggregate of dust cells loaded with intracellular crystals. Mag. × 580.

FIG. 6.—Group of dust cells in alveolar space showing intracellular crystals. Mag. × 1150.

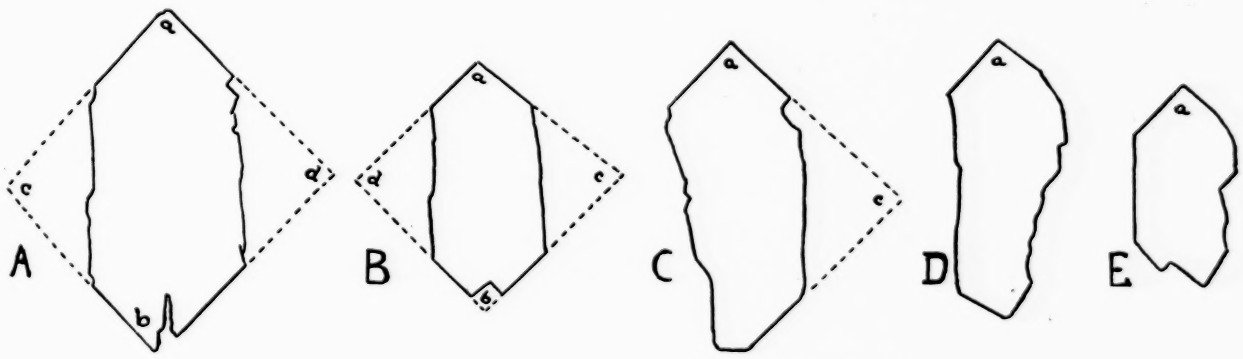


FIG. 7.—Camera lucida drawings of large extracellular plates. Mag. × 750.

tives. They are stained black after Heidenhain's iron-alum hematoxylin process, pink after hematoxylin-eosin-azure (Nocht-Maximow) and eosin-methylene blue. They are not colored by Ziehl-Neelsen's and Gram's stains and are not blackened by osmic acid.

Physical and chemical properties.—The crystals show little or no birefringence either in fixed or in fresh preparations. They are soft enough to cut readily in making paraffin sections.

On heating, the crystals char and gradually disappear, leaving no ash visible by dark field examination. This indicates that their substance is organic rather than inorganic.

The crystals are insoluble in organic solvents (alcohol, ether, ether-alcohol, acetone, and xylene). In fresh suspensions they are insoluble in neutral solutions (distilled water, Ringer's solution, dilute NaCl, half saturated $(NH_4)_2SO_4$) and in boiling water or saline. They are, however, readily soluble in acids of pH between 1 and 2 and in alkalis of pH between 11 and 12. They are dissolved by a saturated solution of Na_2CO_3. In fixed material they are more difficult to dissolve. Concentrated H_2SO_4 and HNO_3 are effective, but concentrated HCl and saturated KOH leave them intact. No swelling takes place in the latter.

Tests for cellulose and lignin (which might be inhaled as dust from the bedding), cholesterol, and uric acid gave results which ruled these out as the substance involved. The xanthoproteic and Millon's reactions were, however, positive. There is strong evidence, therefore, that the substance is a protein. The solubility in dilute acids and alkalis but not in water or dilute neutral salt solutions indicates an amphoteric character. The crystals were soluble in a 6M solution of urea, a trait also of certain proteins as shown by Burk and Greenburg (2). Their persistence in acids and alkalis of concentrations which dissolve them in fresh preparations after such fixing agents as alcohol, formalin, and salts of heavy metals would also be expected of a protein.

The Feulgen reaction was negative. Therefore, while it seems clear that the crystals consist of protein, they probably do not contain in addition thymonucleic acid.

Incidence of Crystals in Swiss Stock Mice

Method of estimating crystal content.—In order to compare the quantities of crystals in the lungs of different individuals, a characterization which will be called the "crystal value" has been worked out. The value for a given mouse is based on a count of dust cells containing crystals in a standard area of tissue in a section chosen at random (30 microscopic fields, 2 mm. oil immersion objective, thickness of section 6 μ). A statistical analysis of the variability of counts within one section and among counts of different sections of the same lung led to the conclusion that 30 microscopic fields was an adequate sample for each mouse. Because of the relative rarity of their occurrence, the large extracellular plates were not included in the counts, nor were crystal plates of intermediate size lying free in the tissue spaces in the few instances in which these occurred (Fig. 4). The number of cells containing crystals in the standard area was then multiplied by a factor based on the typical number of crystals in a single cell (approximately 5 crystals = 1; approximately 10 crystals = 2; approximately 15 = 3; approximately 20 = 4; and intermediate factors 1.5, 2.5, 3.5 accordingly). Since cells which are loaded usually contain more than 20 crystals, and since the crystals tend to increase not only in number but in size, the crystal value errs in the direction of under- rather than of over-estimation of the actual quantity of crystal substance. Although at best the value is a rough approximation to the actual crystal content of the lungs of a given mouse, nevertheless, the degree of uniformity exhibited by mice under similar conditions warrants some confidence in its relative accuracy.

Correlation with age.—Crystals have already begun to appear in mice 3 weeks old and from this time on they are found in increasing quantity. In Table I are listed untreated stock mice between the ages of 5 and 25 months with their crystal values. When the crystal value for each mouse is plotted against its age (Fig. 8) it may be seen that crystal incidence tends to increase with age. From the data in Table I and additional data from untreated control mice used in experiments to be reported later, the coefficient of correlation between crystal value and age for 82 mice of ages ranging from 1 to 24 months was calculated. The value of r_{ca} was found to be 0.64.

Correlation with tumors.—It will be noted from Fig. 8 that for a given age a wide range of crystal values occurs. This suggests that, in spite of a rather high correlation, age alone does not determine the incidence of crystals. It is interesting, therefore, to find another factor with which the amount of crystal substance is associated; namely, the occurrence of tumors.

The Swiss strain of mice has shown an exceptionally high incidence of spontaneous tumors in the lung. Lynch (9) reports 43.9 per cent with tumor among 684 mice of all ages, and in this laboratory the percentage was 61.0 of 159 mice.

An estimation of the volume of tumor in each mouse was made from freehand drawings made at the time of autopsy, and these approximate tumor

volumes are shown in Table I in conjunction with the ages and crystal values.

The relation between the quantity of crystals and the logarithm of the tumor volume is shown graphically in Fig. 9. The age of the mouse is recorded beside each point. Crystal values and ages of mice with no lung tumors are indicated along the vertical axis. This group may be regarded as illustrating the range of normal variation. The correlation between crystal value and tumor volume is closer than between crystal value and age, giving a coefficient (r_{et}) of 0.76.

The coefficient of partial correlation between crystals and tumors, when the age factor is held constant, ($r_{et.a}$), is found to be 0.66, while that between crystals and age, when tumor is held constant, ($r_{ea.t}$), is 0.45.

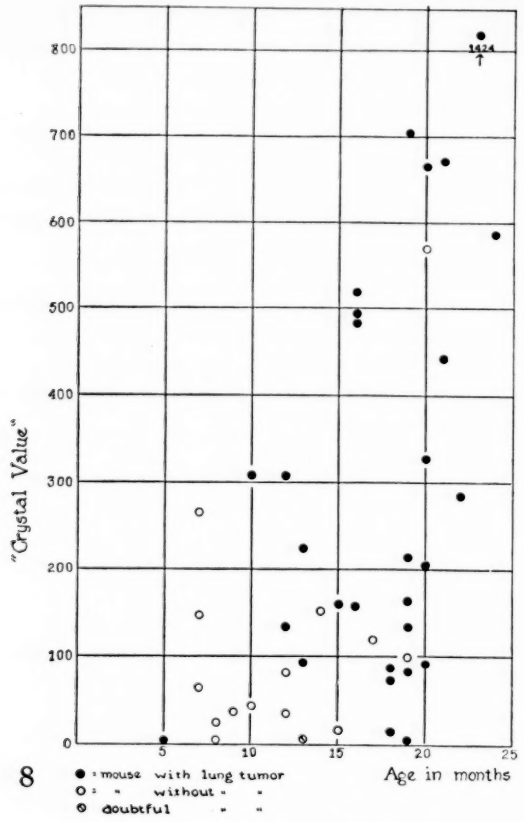
It must be noted that crystals are never found in the tumor cells. The cells which contain them are scattered throughout the lung tissue as they are in normal mice, although there is some tendency for dust cells to accumulate along the margins of the larger tumors.

TABLE I: AGES, APPROXIMATE TUMOR VOLUMES, AND CRYSTAL VALUES OF SWISS STOCK MICE

Mouse	Age in months	Number of nodules	Approximate tumor volume in cu. mm.	Number of cells with crystals in 30 fields	Crystal factor	Crystal value
1	5	1	0.07	4	1.0	4
2	7	0	...	43	1.5	65
3	7	0	...	74	2.0	148
4	7	0	...	89	3.0	267
5	8	0	...	24	1.0	24
6	8	0	...	4	1.5	6
7	9	0	...	25	1.5	38
8	10	0	...	28	1.5	42
9	10	2	4.36	103	3.0	309
10	12	0	...	54	1.5	81
11	12	0	...	23	1.5	35
12	12	3	0.18	103	3.0	309
13	12	1	1.77	53	2.5	133
14	13	4?	0.08?	6	1.0	6
15	13	1	0.52	78	3.0	224
16	13	1	0.07	47	2.0	94
17	14	0	...	76	2.0	152
18	15	0	...	17	1.0	17
19	15	3	0.78	80	2.0	160
20	16	6	172.70	130	4.0	520
21	16	2	0.18	53	3.0	159
22	16	6	15.68	194	2.5	485
23	16	6	2.30	165	3.0	495
24	17	0	...	48	2.5	120
25	18	5	0.78	12	1.0	12
26	18	3	0.55	48	1.5	72
27	18	3	0.07	44	2.0	88
28	19	0	...	40	2.5	100
29	19	4	0.14	82	2.0	164
30	19	8	0.25	54	2.5	135
31	19	2	0.07	108	2.0	216
32	19	3	0.09	5	1.0	5
33	19	2	0.25	56	1.5	84
34	19	3	33.94	176	4.0	704
35	20	0	...	163	3.5	570
36	20	3	9.24	167	4.0	668
37	20	2	0.14	103	2.0	206
38	20	5	9.24	131	2.5	328
39	20	4	1.29	61	1.5	92
40	21	4	30.65	126	3.5	441
41	21	7	54.94	168	4.0	672
42	22	4	8.85	95	3.0	285
43	23	6	56.44	356	4.0	1,424
44	24	6	64.83	147	4.0	588

INCIDENCE OF CRYSTALS IN MICE OF OTHER STRAINS

Examination of slides from mice of other strains showed that the occurrence of crystals is not confined to the Swiss strain. From Table II it may be seen, however, that the incidence of crystals and tumors in these strains is not strictly parallel. While strain



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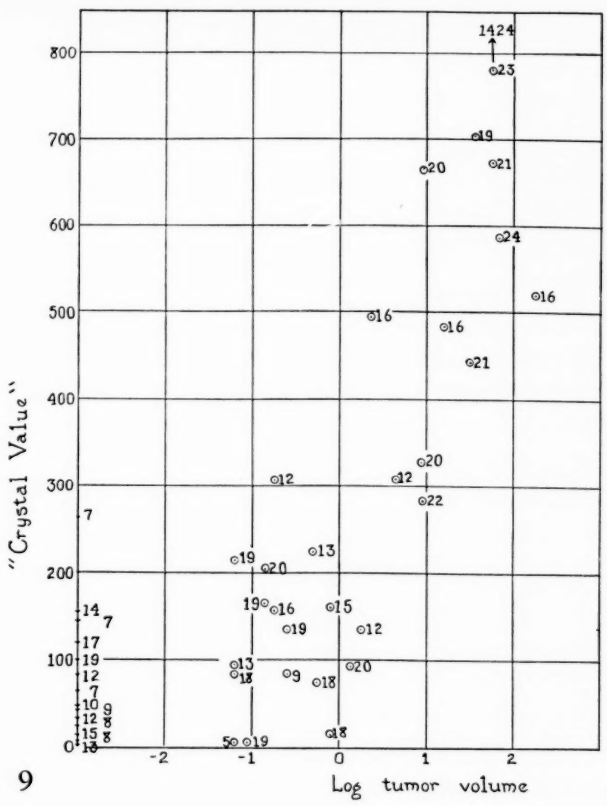

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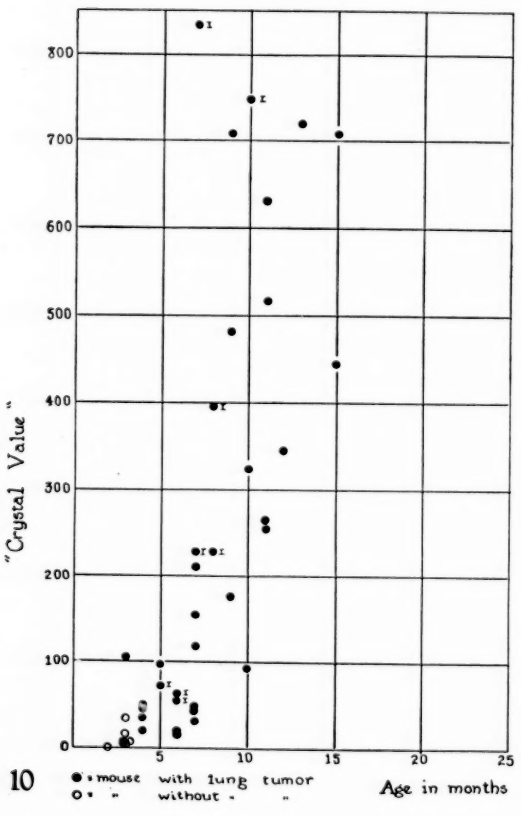

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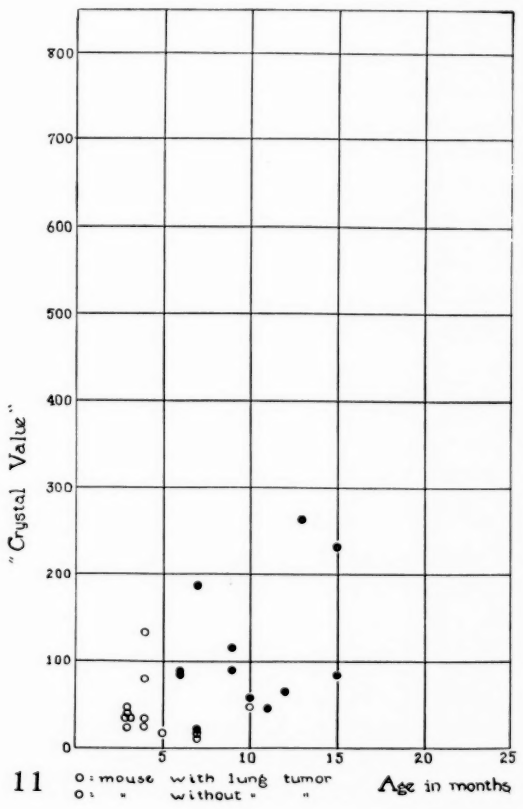

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FIG. 8.—Incidence of crystals in Swiss stock mice.

FIG. 9.—Relation between crystals and tumors in Swiss stock mice.

FIG. 10.—Incidence of crystals in mice treated with 1,2,5,6-dibenzanthracene.

FIG. 11.—Incidence of crystals in untreated control mice.

1194, which is low in lung tumor, shows crystals in only a few individuals, the Bagg albinos and mice of strain A (also partly Bagg in origin) also give low percentages. An additional group of Bagg mice which had been injected with 1,2,5,6-dibenzanthracene showed crystals in 8 out of 10 individuals, which indicates that in these mice, as in the Swiss, dibenzanthracene causes an increase in crystal production. Although the Bagg and A strains give slightly lower lung tumor rates than the Swiss, nevertheless, it is clear that there is not a positive correlation between crystals and susceptibility to lung tumors in untreated mice of these strains.

TABLE II: INCIDENCE OF CRYSTALS IN MICE OF OTHER STRAINS

Strain	Per cent lung tumor	Total mice 10 to 19 months old	Per cent with crystals
Swiss	61.0	27	100.0
A albino	34.0	69	5.8
Bagg albino	31.8 *	39	10.2
1194 agouti	2.4 *	32	9.4

* Lynch (8).

ATTEMPTS TO INFLUENCE THE OCCURRENCE OF CRYSTALS

Experiments with dibenzanthracene.—Since crystal value and tumor incidence appear to be correlated in Swiss mice, tumors were induced by the injection of 1,2,5,6-dibenzanthracene and the effect on the occurrence of crystals observed. Mice 2 to 3 months old were injected subcutaneously in varied locations at intervals of 3 to 8 days. Group I received 5.4 mgm. of dibenzanthracene in lard over a period of 44 days. Groups II to VII received 2.4 mgm. in lard or a commercial vegetable oil over periods of 25 to 40 days. The mice were killed at various times during and after the injection period.

At 54 days after the beginning of treatment every injected mouse had developed multiple tumors whose estimated total volume reached values found in stock mice only after 15 months of age.

The relation between crystal values and age is shown in Fig. 10 for the treated mice and in Fig. 11 for their untreated controls. The two groups gave similarly low crystal values during the first 3 months after the cessation of injections. After this time, the values in the treated mice reached levels not attained by the controls during the period of the experiment, but which correspond to those found in stock mice after 15 months of age.

It will be noted that the development of multiple tumors was well under way before the rise in crystal values occurred. Therefore, while the experiment shows that dibenzanthracene causes an increase in the number of crystals parallel with the increase in lung tumors, the two effects are not simultaneous.

Transmission experiments.—An attempt was made to find out whether or not the occurrence of crystals in the lungs of Swiss mice is under the influence of an agent which can be transmitted from mouse to mouse by inoculation. Various technics were employed to inoculate mice intranasally with suspensions of lung tissue, both filtered and unfiltered.

The inoculated mice showed no outward signs of illness and no deaths occurred. Mice which were allowed to live for periods of one to several months after inoculation gave no significant difference in their crystal values from their uninoculated controls. The incidence of lung tumors in 74 inoculated mice killed at ages ranging from 3 to 8 months was 29.7 ± 3.6 per cent, while their controls gave 25.0 ± 4.9 per cent in 36 mice.

In a considerable proportion (37 per cent) of mice killed 4 to 15 days after inoculation, consolidation of portions of one or more lobes of the lung was found, whereas no visible consolidation was seen in uninoculated mice of similar age. These reactions might be due to the presence of a latent virus such as has been shown to exist in normal Swiss mice by Dochez, Mills, and Mulliken (3) and by Horsfall and Hahn (6, 7). But if the inflammation found in the mice in this laboratory is caused by a virus of this sort it must be one of low virulence, since its activity was not sensibly increased by repeated passage.

DISCUSSION

The cells in which the crystals are found are phagocytic. Several facts, however, suggest that the crystals are formed within the cells. In only a few individuals are crystals found lying free in the tissue spaces. In these cases the neighboring dust cells appear overloaded and bits of protoplasmic debris suggest that some cells have disintegrated, thus releasing their crystals. In young mice, the intracellular crystals are not only few in number but also of small size. In older mice the crystals themselves are larger as well as more numerous. Occasionally a crystal of size comparable to the large extracellular plates has been seen distending the cytoplasm of a single cell.

The fact that plates of the larger type are found in mice only one month old suggests that cells which have accumulated a considerable number of crystals migrate into the lymphatics or bronchi and there form aggregates within which the large plates are laid down. Apparently such cells are not removed from the lung through the normal channels. Groups of them remain beneath the pleura forming hyaline "speckles" visible at autopsy.

It seems evident, too, that the dust cells are unable to destroy the crystals. On the other hand, the crystals cause relatively little damage to the cells which carry

them, for cells with a few crystals have been seen dividing mitotically.

One may next consider what calls out the phagocytic cells. Although macroscopic consolidation of the lungs is rarely found in untreated mice in this laboratory, there is microscopic evidence of a mild inflammatory process in nearly all of the outwardly normal mice. Even in young mice there is a noticeable infiltration of the interstitial tissue with mononuclear cells, the mucous and cellular debris is found in the lumina of the bronchi. The increase in the number of dust cells containing crystals with advancing age is indicative of some continuous disturbance.

If there is an infectious agent responsible for this condition, it is such that its virulence is not greatly enhanced by repeated passage. It is conceivable that the crystals are a by-product or product of decomposition of such an agent, but so far attempts to influence their incidence by transmission have been unsuccessful. While the crystals might be associated with a condition of sensitization or allergy, their properties differ in several respects from those of the Charcot-Leyden crystals as described by Wrede, Boldt, and Buch (13). The mouse crystals may be the result of some simpler upset or deficiency in the normal metabolism of the animal.

Tyzzer (12) suggested the possibility that "the chronic inflammatory changes which are often associated with large numbers of crystals may have some causal relationship to the development of (these) tumors." The reports of several recent workers give no evidence of inflammation associated with the development of pulmonary tumors in mice of strain A (1, 4, 11). The author has as yet made no comparison between the lungs of normal mice of the A and Bagg strains and those of the Swiss strain.[1]

Recently Menkin (10) has found evidence that proliferative changes may be initiated by exudative material. He suggests that a chemical substance released by injured cells over a long period may set up a process which eventually leads to malignancy. This hypothesis seems not without bearing on the situation in the lungs of Swiss mice. That a condition of chronic inflammation exists in these mice seems clear. The reaction is often not apparent with low magnification and only detailed study reveals signs of unusual activity. There is some evidence, however, that the condition can be aggravated by repeated passage to an acute stage, though not to the point of becoming lethal. Further experiments are in progress to determine whether or not the incidence of lung tumors can be influenced by repeated administration of lung extracts over a longer period than has been used in the present series.

Whether or not the crystals are related to the development of tumors is not yet clear. The data from stock mice and those treated with dibenzanthracene show a striking parallelism between crystals and tumors. In both groups, however, tumors make their appearance considerably before the time when the crystals have reached notably high levels. Therefore, if there is a causal relationship, it is probably not the crystals themselves that are responsible but some soluble precursor. A similar conclusion is suggested by the data from the A and Bagg strains, in which crystals appear under normal circumstances in few individuals, yet the tumor rate is high. The fact that in the Bagg mice injected with dibenzanthracene the percentage of individuals showing crystals is considerably higher suggests that the substance may be present in these mice, but in soluble form. Whether or not the substance of which the crystals are composed does play a part in the production of the tumors is yet to be determined.

[1] The slides of the Bagg and A strains examined for crystals were from mice which died from other causes than cancer.

SUMMARY AND CONCLUSIONS

1. A study has been made of crystalline material in the lung tissue of Swiss mice.

2. The crystals are of two main types—large extracellular plates and small plates within the cytoplasm of the "dust cells."

3. Evidence of the crystalline nature of the plates was given by measurement of their angles. Histochemical tests showed that the crystal substance is protein in nature.

4. In Swiss stock mice, the incidence of crystals shows a fairly high correlation with age ($r_{ca}=0.64$) but a still higher correlation with the volume of lung tumor ($r_{ct}=0.76$). High crystal counts are associated with large and frequently malignant tumors.

5. Subcutaneous injection of mice with 1,2,5,6-dibenzanthracene leads to production of both lung tumors and high crystal counts at an earlier age than in control mice. The two effects, however, are not simultaneous. Multiple lung tumors make their appearance several months in advance of any noteworthy increase in crystals.

6. The incidence of crystals in other strains of mice did not show a positive correlation with incidence of lung tumors.

7. Transmission experiments with extracts of lung tissue have so far been without effect on the incidence of crystals. The incidence of lung tumors was nearly the same in the inoculated mice and their uninoculated controls.

8. Outwardly normal stock mice give microscopic evidence of a chronic inflammatory condition in the

lungs with which the crystals are associated. There was some evidence of the presence of an agent causing an acute inflammatory reaction, but it did not become lethal on repeated passage.

9. Suggestions are made concerning the mode of origin of the crystals and their possible relation to inflammation and tumors. No positive correlation between any of the three factors has been established.

This investigation was carried out under the direction of the late Professor David Hilt Tennent, to whom I am deeply indebted for his kindness, advice, and criticism. I wish also to thank Dr. William L. Doyle for his help in interpreting the biochemical aspects of these experiments and Mrs. A. L. Patterson for her help in making the photomicrographs.—AUTHOR.

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The Stimulating Action of Nucleic Acids upon Mouse Heart Fibroblasts*

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This report concerns an extension of previous work dealing with the analysis of extracts of embryonic tissues. It was observed (15) that the macromolecular fraction obtained by sedimentation of the extract in the ultracentrifuge essentially according to Claude (4), stimulated growth of mouse heart fibroblasts *in vitro*. When the macromolecular material was heated in a boiling water bath for 15 minutes the stimulating quality, surprisingly, was found to be augmented. One explanation was that the nucleic acids, which would be liberated under these conditions, might represent the active fraction of the macromolecular complex. The liberation by heat of nucleic acids from macromolecular material has been demonstrated by Claude and Rothen (6). Their relative thermostability in solution is apparent from the method of preparation (13) of the pure substances.

Aside from this problem, the nucleic acids seemed especially worthy of study in tissue culture because of their position in the nucleus and their remarkable physical and chemical properties (10, 12).

MATERIALS AND METHODS

Substances tested.—One preparation of thymonucleic acid (prepared according to Hammarsten) was kindly sent by Dr. Gerhard Schmidt. This formed an opalescent suspension upon long contact with Tyrode's solution and could not be filtered through the Berkefeld N filter. Sterilization was accomplished by immersion in a boiling water bath for 15 minutes. The other nucleic acids, in the form of sodium nucleinates, were obtained from the laboratory of the late Dr. P. A. Levene. The thymonucleinate from this source was stated to have been completely depolymerized. All of these sodium salts formed clear solutions in Tyrode's solution at the concentrations employed and were sterilized by Seitz filtration.

The biotin, as the methyl ester, dissolved in 50 per cent methyl alcohol, was generously furnished by Dr. Vincent du Vigneaud.

The tissue cultures.—The quantitative methods employed in this laboratory have been presented in detail elsewhere (14). The explants of the hearts of newborn mice were grown in Carrel flasks for approximately 72 hours in washed chicken plasma clots with a nutrient medium consisting of rat serum diluted with two volumes of Tyrode's solution. At the end of this preliminary period of growth, the actual experiment was constructed. Usually about 30 colonies were assigned to each of the test and control groups. The test solutions and the Tyrode control were added to each Carrel flask in a volume of 0.3 cc. The nucleinates were dissolved in Tyrode's solution at a concentration of 1:1000. This solution was sterilized by means of a Seitz filter and then appropriately diluted with Tyrode's solution. The diluent solution served as the control.

The areas, A, of the explants were now measured and the cultures were again incubated, for a period of 30 hours. At the end of this experimental interval the areas, B, were determined and the expansion rate factor, E.R.F., for each colony was calculated. This was defined as $\frac{B-A}{B} \times 100{,}000$ divided by 30 (the number of hours in the experimental interval). The statistical significance of the difference of the means of the expansion rate factors was determined by use of Fisher's (9) t factor.

OBSERVATIONS

Effect of heating pellet material.—Evidence additional to the data previously (15) presented that heating the macromolecular material increases its stimulating property is graphically recorded in Fig. 1. The purified macromolecular material (pellet) was obtained by repeated centrifugation of the extract derived from 8-day embryos. Ultracentrifugation at 30,000 r.p.m. (67,000 G.) was alternated with runs at low speed in an ordinary centrifuge to free the preparation of coarsely aggregated material.

* This investigation was aided by grants from The Jane Coffin Childs Memorial Fund for Medical Research and The Anna Fuller Fund.

The stimulating effect of the pellet material heated in the boiling water bath for 15 minutes is apparent. Autoclaving (17 lbs. for 20 minutes) largely destroys the activity of the material; under these conditions some breakdown of nucleic acids is to be expected.

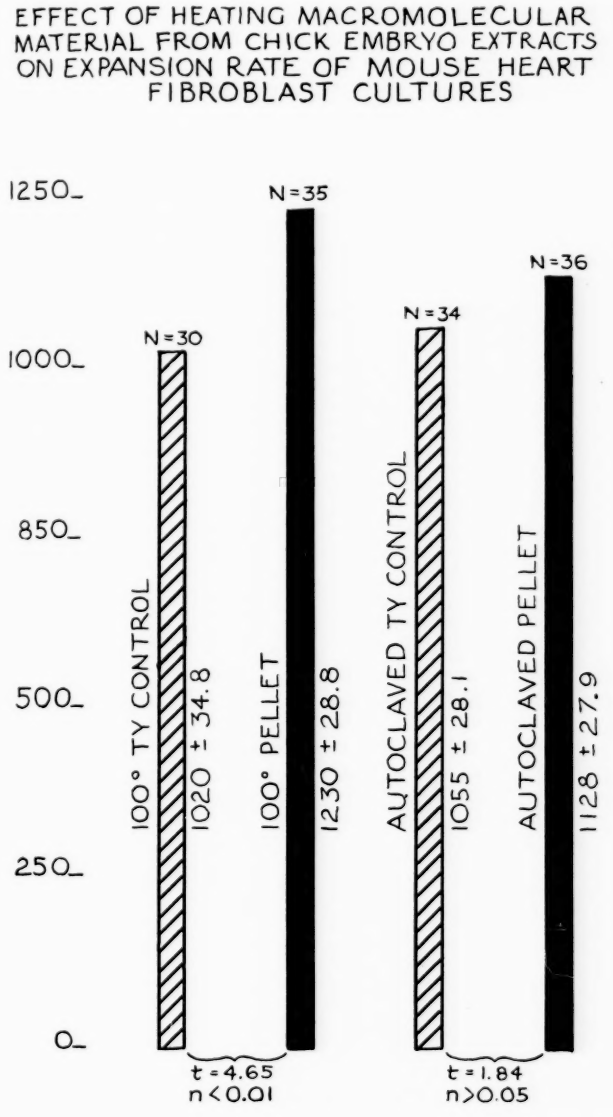

FIG. 1.—The hatched and solid columns are proportional in height to the control and experimental e.r.f. (expansion rate factors, see text), respectively. The numbers on the left are a scale of e.r.f. values. The actual value with its standard error is inscribed at the right of each column. N = number of colonies under observation in each group; t = Fisher's (9) factor; n = probability of error.

Effect of pure nucleic acid and sodium nucleinates.—Of 15 series of observations with 5 different preparations, 6 showed statistically significant degrees of stimulation at certain concentrations, and in 8 the expansion rate factors of the nucleic acid-treated cultures were greater than those of the controls, but not by a statistically adequate margin. The one exception is explained in the description of Table IV. The individual data are summarized in Tables I to V.

It is not possible from these experiments to state which of the nucleinates has the greatest activity, since one of the essential characteristics of the method is that only simultaneous observations under the same conditions are valid in making comparisons. In the case of any particular nucleinate a concentration in the medium of 1:25,000 to 1:50,000 seems optimal, although in some experiments with nucleinates derived from pancreas and fish sperm, concentrations as low as 1:250,000 were still effective.

In one experiment with fish sperm nucleinate (Table IV, experiment 342) the test and control solutions were boiled under the same conditions as the pellet. A high degree of activity was retained. This is direct evidence of the stability of the nucleinate.

The effect of biotin.—Since vitamin H, whose identity with biotin is receiving increasing experimental confirmation (11), is liberated from liver by prolonged autoclaving, the possibility that the increased activity of the macromolecular material following heating might be the result of release of this material, was considered. Consequently the biotin methyl ester, originally in a concentration of 5 γ per cc. in methyl alcohol, was diluted with Tyrode's solution and added to the medium so that the final concentration was 1:200,000,000 and 1:100,000,000 respectively in each of two experiments. Methyl alcohol was diluted with Tyrode's solution and added to the control colonies to attain the same concentration as in the experimental cultures, 1:200 and 1:100 respectively. No significant result was obtained. The lack of effect with this increment of biotin may be on account of the fact that the culture medium is probably rich in such constituents.

DISCUSSION

These observations confirm the theory that nucleic acids released from the stimulating macromolecular material of chick embryo extract may account for the increase in its activity after heating. It can be calculated from the concentration of the macromolecular material in the extracts employed (approximately, 1.5 per cent) and from its content in nucleic acids (approximately 16 per cent) that enough of the latter is present to produce a concentration in the culture medium in excess of 1:10,000 provided that the nucleic acids were completely released by thermal or enzymatic action.

Carrel (2) had previously found the activity of embryo extract to diminish upon heating to 56° C. for 10 minutes and to be completely lost when heated for the same length of time at 70° C. This may be accounted for by the denaturation and precipitation

TABLE I: YEAST RIBONUCLEINATE *

Experiment number	Concentration	E.R.F. Tyrode control	E.R.F. nucleinate	*t*	*P*
328	1:10,000	1189 ± 28.7 (34)	1230 (32)	...	N.S.
	1:50,000	.....	1294 ± 24.8 (36)	2.77	<.01
	1:250,000	.....	1203 (40)	...	N.S.
333	1:10,000	973 ± 47.5 (24)	963 (34)	...	N.S.
	1:50,000	.....	1029 (28)	...	N.S.
	1:250,000	.....	1109 ± 37.7 (32)	2.24	<.05

* Key: E.R.F. = Expansion rate factor (see text).
t = Fisher's (9) *t* factor.
P = Probability of error.
N.S. = Not significant.

The figures in parentheses indicate the number of colonies in the group under observation.

TABLE II: THYMUS THYMONUCLEINATE *

Experiment number	Concentration	E.R.F. Tyrode control	E.R.F. nucleinate	*t*	*P*
329	1:50,000	1290 ± 35.8 (31)	1401 ± 24.3 (35)	2.56	<.02
	1:250,000	.....	1337 (29)	...	N.S.
	1:1,250,000	.....	1298 (31)	...	N.S.
332	1:12,500	993	998	...	N.S.

* For abbreviations, see footnote to Table I.

TABLE III: PANCREAS NUCLEINATE *

Experiment number	Concentration	E.R.F. Tyrode control	E.R.F. nucleinate	*t*	*P*
334	1:10,000	1119 ± 39.7 (31)	1199 (35)	...	N.S.
	1:50,000	.....	1309 ± 22.0 (33)	4.18	<.01
	1:250,000	.....	1273 ± 26.2 (36)	3.23	<.01
336	1:50,000	1103 (27)	1097 (38)	...	N.S.
	1:250,000	.....	1157 (32)	...	N.S.
337	1:50,000	1250 (29)	1298 (35)	...	N.S.
	1:250,000	.....	1221 (25)	...	N.S.
338	1:25,000	946 ± 40.1 (26)	1146 ± 35.1 (28)	3.76	<.01
	1:100,000	.....	1037 (30)	...	N.S.

* For abbreviations, see footnote to Table I.

of the ordinary proteins and the mechanical "dragging down" or "adsorption" of the macromolecular material. Such factors may account also for the apparent thermolability of the nucleoprotein which Fischer (8) identified as the growth-stimulating principle of beef embryo extract. In the pure state the macromolecular substance is not precipitated from neutral solutions by heating (5) although partial decomposition takes place.

in macromolecular material, nor that the latter is the sole active material in embryo extract. The work of Carrel and Baker (3) as well as that of Fischer (7) upon the proteins of the embryonic extract and upon the higher peptic digestion products of proteins, points to the existence of other factors.

It is suggested that the nucleinates offer the cells a metabolic short-cut so that growth is more rapid. By this is meant that nucleogenic material is supplied

TABLE IV: FISH SPERM NUCLEINATE *

Experiment number	Concentration	E.R.F. Tyrode control	E.R.F. nucleinate	*t*	*P*
340	1:50,000	1077 ± 34.1 (28)	1150 ± 36.1 (29)	1.67	N.S.
	1:250,000	.....	1098 (23)	...	N.S.
341	1:50,000	1316 ± 27.0 (39)	1355 ± 31.4 (29)	1.75	† See Note
	1:250,000	.....	1217 ± 35.8 (31)	3.10	† See Note
342	1:50,000	1250 ± 27.4 (35)	1374 ± 29.2 (38)	3.10	<.01
	1:250,000	.....	1329 ± 28.0 (35)	2.02	N.S.
356	1:10,000	1259	1308	...	N.S.
	1:50,000	.....	1273	...	N.S.

* For abbreviations, see footnote to Table I.
† Note: In experiment 341 it is probable that the control and 1:250,000 solutions were accidentally exchanged before they were added to the cultures. These solutions were applied by the workers not concerned with measuring the colonies in order to avoid possible bias. If there actually has been an error in labelling, then both the 1:50,000 and 1:250,000 solutions show a striking stimulating effect.

TABLE V: NUCLEIC ACID (SCHMIDT) *

Experiment number	Concentration	E.R.F. Tyrode control	E.R.F. nucleinate	*t*	*P*
339	1:100,000	1267 ± 32.6 (33)	1343 ± 27.1 (28)	1.80	N.S.
344	1:50,000	1313 (24)	1381 (26)	1.45	N.S.
345	1:50,000	1188 (27)	1222 (34)	...	N.S.

* For abbreviations, see footnote to Table I.

The similarity in range of effective concentrations with the various nucleinates is of interest. Baker and Carrel (1) state that pure nucleoprotein and nucleic acid were without stimulating effect upon chick heart fibroblasts. The factor of concentration may account for the discrepancy in results. Nucleinates at concentrations in excess of 1:10,000 in our experience showed no stimulating action or had an inhibitory effect. In concentrations below 1:10,000 the nucleinates were devoid of significant effect upon the pH or osmotic pressure of the medium.

It cannot be claimed from these experiments that the nucleinates represent the only stimulating factor in larger chemical blocks (nucleinates or their digestion products) than are ordinarily available in the medium for synthesis.

SUMMARY AND CONCLUSIONS

Sodium nucleinates from various sources significantly increase the expansion rates of mouse heart fibroblasts at concentrations in the medium of about 1:50,000. This is evidence that the nucleinates represent one stimulating factor of embryo extract or its derivative macromolecular material. The thermal or enzymic breakdown of the macromolecular material

is calculated to yield at least the effective concentration of nucleinates.

These observations do not intimate that other components of embryonic extract may not also have a stimulating action.

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Hemoglobin Level and Tumor Growth*

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The association of anemia with cancer has been reported for many different types of animals. It has been repeatedly observed that the later stages of malignant growths are characterized by a severe and progressive depression in the blood hemoglobin level. This has been found to be true of many different types and locations of cancer in man, mice, rats, rabbits, birds, etc. (1, 2, 4, 5, 9, 13, 15).

In reporting their observations of this effect most workers have described it as a "secondary anemia" and in general consider it to be the result of the general toxic action of the cancer on the host. Such conditions as hemorrhage, infection, the presence of necrotic tissue, and general debilitation have been suggested as mediating factors (1, 3, 14).

The possibility that cancerous growths may have a direct and destructive effect on hemoglobin or inhibit its production has not apparently been seriously considered.

It has been recently shown by Craig *et al.* (8) that the concentration of cytochrome C in cancer tissue is lower than in normal tissue. Greenstein and co-workers (10) also recently reported that the introduction of liver tumor tissue into an animal produced a very rapid and striking decrease in the liver catalase concentration. Both of these observations indicate that cancer tissue has an antagonistic action on enzyme systems of this type and since both cytochrome and catalase enzymes have the same type of hemin prosthetic groups as hemoglobin, it would not be surprising to find that hemoglobin was acted upon in the same manner by tumorous tissue. These considerations could conceivably account for the striking anemia characteristic of the later stages of cancer, which is referred to above. However, in the advanced period it is difficult to dissociate the primary from the secondary effects of tumor growth. For that reason the problem necessitates the study of the precancerous and early cancerous conditions. Under these circumstances, significant decreases in the blood hemoglobin concentration should constitute fairly valid indications of an antagonism between cancer tissue and hemoglobin.

Strong has painted a clear picture of the blood hemoglobin in relation to inherited cancer susceptibility and resistance in mice. In a series of investigations (16-19) he has shown that the hemoglobin level of mice of high spontaneous tumor incidence falls off markedly long before cancerous growths appear, in contrast to cancer-resistant strains which do not react in this manner. However, he considers this genetically-determined effect to be unrelated to the anemia which appears after cancer develops and which he speaks of as being secondary in nature. So far as can be ascertained, no one has investigated the effect of a chemically-induced precancerous stage on the hemoglobin level.

In studies which utilized several varieties of rabbits, Casey, Pearce, and co-workers reported (6, 7, 11) that the resistance of these animals to implanted malignant tumors was related to the hemoglobin level which prevailed before tumor inoculation. They found that the most resistant animals were those with "normal" blood hemoglobin concentrations and red blood cell counts while rabbits in which these values were relatively high or low proved to be more susceptible to the growth of the tumor implants. They also investigated the reactions of the blood to the growth of transplanted cancer tissue. Their results disclosed no appreciable change in hemoglobin concentration or red blood cell counts for the early stages of tumor development.

Recently Blumenthal (3) reported the results of an investigation of the reaction of blood cellular elements to the growth of neoplastic implants. Several types of tumors and both mice and rats were utilized. He found, among other things, no significant change in the red cell count while the tumors were comparatively small. Anemia developed after the tumors had attained a large size and the animals had become debilitated.

A number of other papers might be cited since the reactions of the blood to cancer have long been and still are a fruitful field for research. But as indicated before, the relation of cancerous growth to the hemoglobin level has been generally regarded as secondary. Such data as can be found in the literature have been obtained in studies which were primarily concerned with other aspects of cancer-blood relationships.

Preliminary work in our laboratory indicated the possibility of a direct relation between the hemoglobin level and not only the early stages of cancer, but also the chemically induced precancerous condition.

MATERIALS AND METHODS

All hemoglobin determinations were made by the method described by Evelyn using an Evelyn photoelectric colorimeter. This technic proved to be sensitive and thoroughly reliable.

Tumor implants.—The method of hypodermatic injection of finely divided tissue was used. The tumor tissue was forced through muslin, the resulting material passing easily through a No. 18 needle. Each animal received 0.05 ml. of this undiluted homogeneous tissue subdermally in the mid-dorsal area posterior to the cervical vertebrae.

Mammary gland adenocarcinomas which had become stabilized by many generations of transplants furnished the material for the implants made into the Dba group. The C57 mice received portions of a round cell sarcoma which had originally been produced by the action of methylcholanthrene in this

* This investigation was generously supported by the Clayton Foundation, Houston, Texas.

strain. Only animals without ulcers or any sort of external lesions were included in the experiment. The sarcoma implants used for the rats were also obtained from a tumor induced by methylcholanthrene.

Cancer induction by butter yellow.—White rats of the Wistar strain were fed on two basal diets, each of which contained 0.6 per cent of dimethylaminoazobenzene (butter yellow) incorporated as a 3 per cent solution in olive oil. Diet 1 consisted of Purina dog chow, and diet 2 was cooked whole rice plus raw carrots and salt.

Cancer induction by methylcholanthrene.—Dba and C57 mice received single injections of 0.4 mgm. of methylcholanthrene dissolved in 0.2 ml. of olive oil subcutaneously in the right axilla.

Fig. 3 show clearly the striking decrease in hemoglobin produced in rats when butter yellow (*p*-dimethylaminoazobenzene) was added to the diet. When the basal diet was cooked whole rice and raw carrots, the decrease in hemoglobin content was about 24 per cent in 30 days. At this period the liver of these animals was macroscopically unaffected. Nodular cirrhosis affected the livers of most of the animals before the end of the 148-day period. As the figure shows, the hemoglobin level remained almost constant after the initial drop. With the development of hepatoma nodules, the hemoglobin level fell below 11 gm. per cent or 36 per cent below normal.

The use of a nutritionally more complete basal diet (Purina dog chow) led to an initial drop of about 12.5 per cent in 30 days. Thereafter the level remained almost constant as far as has been observed (162 days).

TABLE I: THE EFFECT OF GROWTH OF TUMOR IMPLANTS ON THE BLOOD HEMOGLOBIN CONCENTRATION

Experiment	Animals: Strain	Animals: Number	Tumor	Days after implantation	Tumor size *	Hemoglobin (gm. per 100 ml.)	Relative hemoglobin values † (controls = 100)
1	Dba mice	15	Adenocarcinoma	7	0.5 cm.	15.1 ± 0.7	93.0
				14	1.5 cm.	14.2 ± 0.9	88.0
				21	3.0 cm.	8.4 ± 0.6	52.0
2	Dba mice	140	Adenocarcinoma ‡	7	0.59 ccm.	15.1	88.9
				14	3.85 ccm.	12.8	75.2
				21	13.30 ccm.	6.7	39.7
3	C 57 mice	17	Sarcoma	20	2.3 cm.	10.2 ± 1.1	62.5
4	Wistar rats	6	Sarcoma	..	4.5 cm.	9.5 ± 2.5	55.7

* The values for experiment 2 were obtained by multiplying diameter measurements in three dimensions directly. In the other experiments, the average diameters of measurements in two dimensions only are recorded.
† The average hemoglobin values of the animals before implantation were used as controls.
‡ Each animal received 0.02 cc. of original cancer tissue diluted to 0.2 cc. with sterile saline solution, instead of the usual 0.05 cc. of undiluted tissue.

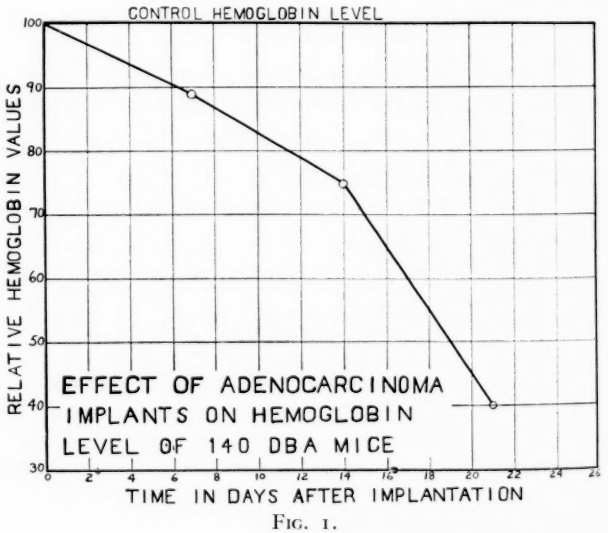
RESULTS

Tumor implants.—The decided drop in hemoglobin level effected by the implantation of tumor tissue in Dba mice is clearly shown in Table I and Fig. 1. At the end of the first week, the tumors were very small, and the animals were vigorous and healthy; nevertheless, 7 to 10 per cent hemoglobin decreases had occurred. This trend was continued during the second week, and accelerated with time until death occurred.

The same general degree of effect was observed with the sarcoma implants in C57 mice and Wistar rats.

Methylcholanthrene injections.—The results (Table II, Fig. 2) show that a 4 per cent average drop in hemoglobin had occurred in 22 Dba mice 50 days after injection of the methylcholanthrene. That this drop was real was borne out by the fact that the hemoglobin level continued to fall to a level approximately 10 per cent below normal by the time 83 days had elapsed. This same effect was observed with the C57 mice which had received methylcholanthrene implants. No visible tumors were evident at this time, but after 103 days, 21 per cent of the animals showed visible tumors, and the hemoglobin level had fallen to 84 per cent.

Effect of butter yellow and diet.—Table III and


CONTROL HEMOGLOBIN LEVEL
RELATIVE HEMOGLOBIN VALUES
EFFECT OF ADENOCARCINOMA IMPLANTS ON HEMOGLOBIN LEVEL OF 140 DBA MICE
TIME IN DAYS AFTER IMPLANTATION
FIG. 1.

DISCUSSION

As the results show, there was a definite drop in the hemoglobin level in the very early stages of the growth

of tumor implants. The data obtained from 155 mice left little room for doubt on this point. Records on 25 mice for which the changes in hemoglobin level were followed individually (although not reported individually here) manifested in each individual case a decline in the hemoglobin concentration even before the tumors were large enough to be measured. The later stages of tumor development were associated with an ever-decreasing hemoglobin level. Some mice showed a fall in this respect from 17 gm. per cent to less than 3 gm. per cent before death.

While factors other than the growth of the cancer tissue may play a part in the extreme anemia which developed in the later stages, it is unlikely that such an explanation could be reasonably advanced for the initial moderate hemoglobin depression which accompanied the early development of the tumor. The animals at this time appeared vigorous and unimpaired.

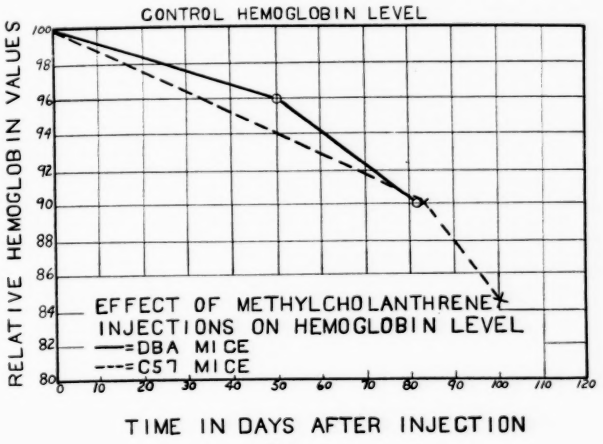


FIG. 2

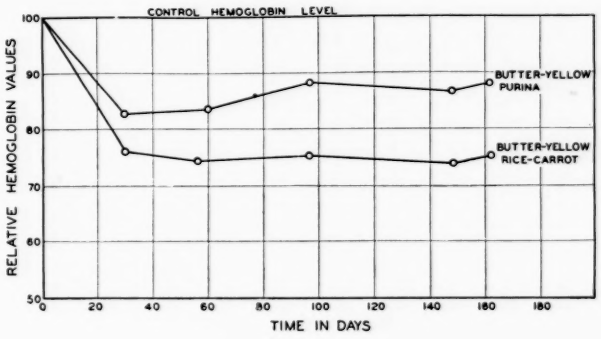


FIG. 3

TABLE II: THE EFFECT OF SUBDERMAL DEPOSITS OF METHYLCHOLANTHRENE ON THE BLOOD HEMOGLOBIN CONCENTRATION

Experiment	Mice: Strain	Mice: Number	Days after injection	Per cent bearing visible tumors	Hemoglobin (gm. per 100 ml.)	Relative hemoglobin values (controls = 100)
1	Dba	22	50	0	15.6 ± 0.6	96.0
			83	0	14.7 ± 0.7	90.0
2	C57	19	84	0	15.3 ± 1.0	90.2
			103	21	14.3 ± 1.2	84.3

TABLE III: THE EFFECT OF BUTTER YELLOW INGESTION ON THE BLOOD HEMOGLOBIN LEVEL OF THE RAT

Time on diet, days	Number of animals	Average weight, in gm.	Hemoglobin, gm. per cent	Relative hemoglobin values (controls = 100) *
1. PURINA DOG CHOW + 0.6% BUTTER YELLOW				
0	9	154.4	15.4 ± 0.7	100.0
30	9	171.8	13.5 ± 0.8	82.7
56	9	176.3	13.6 ± 0.8	83.5
97	9	180.6	14.4 ± 0.7	88.3
148	9	177.3	14.1 ± 0.9	86.6
162	9	175.6	14.4 ± 0.3	88.3
2. COOKED WHOLE RICE, RAW CARROTS, + 0.6% BUTTER YELLOW				
0	8	168.5	16.3 ± 0.9	100.0
30	8	161.5	12.4 ± 0.3	76.2
56	8	152.7	12.1 ± 0.6	74.5
97	8	154.9	12.3 ± 0.7	75.6
148	5	165.5	12.0 ± 0.5	73.6
162	5	165.7	12.3 ± 0.6	75.5

* All values based on 16.3 gm. per cent of hemoglobin as 100, except for the first value reported, which was based on 15.4 gm. per cent. In rats of that age and size the hemoglobin level is still rising.

The hemoglobin reaction to the methylcholanthrene- and dimethylaminoazobenzene-produced precancerous states was also striking in its uniformity. The effect of the methylcholanthrene deposits on the hemoglobin level appeared to be not unlike the effect in this regard of the genetically determined influence which Strong (19) reported as operating in cancer-susceptible mice. It might well be that the two phenomena are closely related, perhaps differing mainly in the intensity of the carcinogenic stimulus which of course is more intense in mice receiving the chemical.

It is especially worthy of note that the cancer-resistant and the cancer-susceptible mice reacted in the same manner to the treatment with methylcholanthrene. As the results show, a group of C57 black mice and a group of Dba mice each manifested a hemoglobin depression of 10 per cent by about the 80th day, before visible tumors had appeared, after receiving the methylcholanthrene deposits. A carcinogen of this type is so powerful that genetic influences tend to be nullified.

The effect of butter yellow ingestion on the blood hemoglobin of rats probably involves some factors not present in the action of methylcholanthrene. As the

data show, the hemoglobin in this instance dropped suddenly to a new level and then remained constant until the appearance of hepatomas at which time a further drop was recorded. Liver damage does not seem to be a satisfactory explanation since the livers at the 30-day period were macroscopically normal, while in the later period severe cirrhosis developed. However, the hemoglobin concentration remained stabilized at the new level and was apparently unaffected by the progressive damage to the liver tissue. As time went on, there was actually less individual variation in the hemoglobin levels of the individual rats in this experiment.

In this connection, it was significant that a diet (rice-carrot) known to favor the development of liver carcinoma in the presence of butter yellow also effected a more severe decline in the hemoglobin level.

Rats, fed butter yellow, which developed hepatomas did not manifest the extreme anemia which was characteristic of the late stages of external tumors. Subdermal implants of adenocarcinoma may attain a comparatively large size before killing the animal; sometimes as much as 20 gm. of tumor tissue develops in a 30 gm. mouse. Under such circumstances the hemoglobin level may be depressed 85 per cent below normal. Obviously, a liver tumor would cause death long before attaining any such size and, accordingly, the late stages of hepatoma tend to depress the hemoglobin level to a lesser extent; *i.e.,* about 36 per cent below normal.

The underlying mechanism responsible for the results reported here must necessarily remain obscure until this aspect of the problem has been thoroughly investigated. However, it does seem permissible, especially in view of Craig's and Greenstein's work (8, 10) referred to before on the effect of cancer on cytochrome C and catalase, to postulate an antagonistic action between hemoglobin and cancerous tissue, and to a lesser extent precancerous tissue. As far as the precancerous condition is concerned, it seems probable that one of the characteristics of cancer tissue which is gradually assumed by the cells undergoing transformation is that of incompatibility with high hemoglobin levels. It is likely that this is true of mice which have a tendency towards spontaneous cancer as well as animals under the influence of applied chemical carcinogens.

SUMMARY AND CONCLUSIONS

1. Evidence based on results obtained from a study of 155 tumor implants in mice indicated that tumor tissue has an antagonistic action toward the blood hemoglobin of the host. The hemoglobin level was depressed by the time the implant had grown to measurable size and thereafter became progressively lower until death occurred.

2. The precancerous condition as induced by methylcholanthrene was associated with a gradual fall of hemoglobin values which began soon after the mice received injections of this chemical and continued on into the cancerous condition. The hemoglobin of cancer-susceptible and cancer-resistant mice behaved in the same manner to this carcinogen. It was concluded from these results that precancerous tissue was more or less antagonistic to hemoglobin in the degree to which it approached the cancerous state.

3. Ingestion of butter yellow by rats induced a sudden drop in the hemoglobin to a new level by the 30th day of the experiment. Thereafter the hemoglobin concentration remained fairly constant until hepatomas developed, after which a further decline was noted. The hemoglobin of butter yellow-fed rats dropped to a much lower level (76 per cent) on a rice-carrot basal diet then on a Purina dog chow basal diet (83 per cent). Increasing liver damage as manifested by the appearance of cirrhotic nodules did not effect any further change in the hemoglobin level over a 162-day period.

We wish to acknowledge the invaluable assistance and encouragement of Professor Roger J. Williams, Director of the Biochemical Institute. Our thanks are also due to Mrs. Dorothy Pennington and Miss Juanita Thacker for technical assistance rendered during the course of this research.—*Authors.*

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Effect of Extirpation of the Tumors. J. Biol. Chem., **141**:327-328. 1941.

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Correction

See: Kensler, C. J., S. O. Dexter, and C. P. Rhoads. The Inhibition of a Diphosphopyridine Nucleotide System by Split Products of Dimethylaminoazobenzene. Cancer Research, **2**:1-10. 1942.

In the graphic formula for the split product of compound III in Fig. 7, p. 9, the methyl group shown in the ortho- position should be in the meta- position.

Abstracts

Reports of Experimental Research

Carcinogenic Compounds

KENNAWAY, E. L., N. M. KENNAWAY, and F. L. WARREN. [Roy. Cancer Hosp. (Free), London, England] THE EXAMINATION OF DIMETHYLANTHRACENES FOR CARCINOGENIC PROPERTIES. Cancer Research, 2:157-159. 1942.

Five dimethylanthracenes (1,2-, 1,3-, 1,4-, 2,3-, 9,10-) were tested for carcinogenic activity by application to the skin, and by subcutaneous injection and implantation, in mice. The 9,10- compound alone produced epithelioma of the skin, and adenomas of the lung were most abundant in the mice receiving this compound. None of the 5 compounds produced tumors of the subcutaneous tissue. The results indicate: 1. the importance of substitution in the 9,10- positions as a source of carcinogenic activity, and 2. the inadequacy of subcutaneous injection as a sole test for carcinogenic power.—Authors' summary.

LAVIK, P. S., P. R. MOORE, H. P. RUSCH, and C. A. BAUMANN. [Univ. of Wisconsin, Madison, Wis.] SOME ADDITIVE EFFECTS OF CARCINOGENIC HYDROCARBONS. Cancer Research, 2:189-192. 1942.

Of 586 mice which developed tumors as a result of the application of methylcholanthrene for 2 months, 92% did not develop them until after the carcinogenic treatment had been discontinued.

Methylcholanthrene was applied to the skin of mice in amounts insufficient to produce tumors in the majority of the animals, and methylcholanthrene, benzpyrene, dibenzanthracene, or benzanthracene were then applied for relatively short periods of time. Benzanthracene failed to increase the incidence of tumors, but the other hydrocarbons exerted additive effects which were equivalent to the known carcinogenic potencies of the compounds for mouse skin.

When methylcholanthrene was applied to the skin of mice for 2 months, and intervals up to 4 months allowed before the application of hydrocarbon was resumed for 1 month more, many tumors developed as a result of the repainting procedure.

These observations suggest that the initial application of hydrocarbon had produced subcarcinogenic alterations in the tissue. An attempt has been made to correlate the results with an hypothesis that carcinogenesis is essentially an accumulation of abnormal protein within the cell.—Authors' abstract.

STEINER, P. E. [Univ. of Chicago, Chicago, Ill.] ABSENCE OF CARCINOGENICITY OF A COD LIVER OIL CONCENTRATE. Cancer Research, 2:181-182. 1942.

A cod liver oil concentrate prepared by saponification and extraction with ethylene dichloride has not been carcinogenic when injected into mice.—Author's summary.

STOWELL, R. E., and W. CRAMER. [Barnard Free Skin and Cancer Hosp., and Washington Univ. Sch. of Med., St. Louis, Mo.] THE EFFECT OF SOLVENTS IN METHYLCHOLANTHRENE EPIDERMAL CARCINOGENESIS. A COMPARISON OF BENZENE AND ACETONE. Cancer Research, 2:193-197. 1940.

Three series of female Swiss mice, totaling 123 animals, were painted on a large area of the skin of the back with methylcholanthrene dissolved in acetone and in benzene, respectively. The carcinogenic response to a 0.3% solution of methylcholanthrene was better in acetone than in benzene solvent. Mice painted with a 0.3% solution of methylcholanthrene in acetone 3 times a week and with pure benzene on alternate days developed cancer even more rapidly.

The application of solutions to the ears of a rabbit showed that the hyperemia after the application of methylcholanthrene in benzene was a combined effect of the carcinogen and of its solvent. The less pronounced hyperemia after the application of methylcholanthrene in acetone was caused by the carcinogen alone, since pure acetone did not produce appreciable hyperemia.—Authors' abstract.

Hormones

BISCHOFF, F., M. L. LONG, J. J. RUPP, and G. J. CLARKE. [Santa Barbara Cottage Hosp. Research Inst., Santa Barbara, Calif.] INFLUENCE OF TOXIC AMOUNTS OF ESTRIN UPON INTACT AND CASTRATED MALE MARSH-BUFFALO MICE. Cancer Research, 2:198-199. 1942.

Estradiol in sesame oil was administered in a total dose of 3.5 mgm. per mouse intermittently over a 12-month period to groups of castrated and intact male Marsh-Buffalo mice. In the castrated mice the incidence of lymphosarcoma was increased significantly above that occurring in both estrin-treated and control intact mice. No mammary gland tumors appeared in the series of intact males and only 6 per cent (2 tumors) developed in the castrated group, which had received estrin. To date carcinoma of the breast has not been produced in intact male Marsh-Buffalo mice by the administration of estrogens over a long period of time either in toxic or nontoxic doses.—Authors' abstract.

LIPSCHÜTZ, A., J. V. LUCO, and J. ZAÑARTU. [Nat. Health Service, Republic of Chile, Santiago, Chile] QUANTITATIVE ASPECTS OF THE ANTIFIBROMATOGENIC ACTION OF SYNTHETIC DESOXYCORTICOSTERONE ACETATE. Cancer Research, 2:200-203. 1941.

When tablets or pellets of estradiol and synthetic desoxycorticosterone acetate are implanted simultaneously under the skin of the female guinea pig, the fibromatogenic action of the estrogen can be inhibited by equimolecular quantities of the adrenal cortical steroid. This holds only for sufficiently high levels of the fibromatogenic estrogen; at lower levels the equimolecular ratio often loses its inhibitory action and the quantity of the antifibromatogenic steroid necessary to prevent fibroids is then a multiple of the tumorigenic one. This may be explained by the assumption that the inhibitory effect depends also

upon a threshold dosage of the antitumorigenic steroid. Ineffectiveness of very small pellets may depend also on a premature more or less complete absorption of the source of the antifibromatogenic steroid.—Authors' summary.

LIPSCHÜTZ, A., O. VERA, and S. GONZÁLEZ. [Dept. of Exper. Med., Nat. Health Service, Republic of Chile, Santiago, Chile) THE RELATION OF THE ANTIFIBROMATOGENIC ACTIVITY OF CERTAIN STEROIDS TO THEIR MOLECULAR STRUCTURE AND TO VARIOUS ACTIONS OF THESE HORMONES. Cancer Research, 2:204-209. 1942.

The antifibromatogenic threshold, or the minimum quantity which must be released per day by a subcutaneous tablet of the steroid so as to inhibit fibroids, was lowest with progesterone; desoxycorticosterone (acetate) was next in order; testosterone (propionate) was highly active but less than progesterone and desoxycorticosterone. Androstanediol (propionate) had no antifibromatogenic action.

The diminution of the average tumorigenic effect and other quantitative criteria of antifibromatogenic action were in accord with the statements referring to the antifibromatogenic threshold.

The descending order of antifibromatogenic faculty of different steroids was coincident with their antiestrogenic activity in the guinea pig, but not in the rat. The masculinizing activity was not coincident with the descending order of antifibromatogenic action. There was coincidence between the descending order of antifibromatogenic activity as studied in the guinea pig and the progestational activity in the rabbit. Species differences prevent generalization of these results.

Antifibromatogenic activity of steroids is apparently reinforced by the ketonic oxygen in position 3 or by the double bond at position 4-5 in ring I, or by both of these, and by carbons attached in position 17 (the short side chain of carbons 20 and 21).—From authors' summary by S. B-J.

Genetics

HESTON, W. E. [Nat. Cancer Inst., Bethesda, Md.] RELATIONSHIP BETWEEN SUSCEPTIBILITY TO INDUCED PULMONARY TUMORS AND CERTAIN KNOWN GENES IN MICE. J. Nat. Cancer Inst., 2:127-132. 1941.

Strain A mice were crossed with strain W mice containing the shaker-2, waved-2, and flexed-tail genes. The F_1 generation of this mating were backcrossed with the parental strain W mice. The resulting generation was classified as to whether the mice were shaker or normal, waved-coated or normal-coated, or flexed-tailed or normal-tailed. These animals were all inoculated with 20-methylcholanthrene and killed for observation 16 weeks later. The mice bearing the normal alleles of shaker-2 and waved-2 genes displayed a higher incidence of induced pulmonary tumors than the shaker-waved group. A similar but more striking difference was observed between the shaker animals and the nonshaker animals irrespective of the waved-2 gene. The nonshakers bearing the normal allele from the A strain showed higher susceptibility than the shakers. On the other hand, if they were classified as to the waved-2 gene, the normals and waved-2 cross showed about the same susceptibility. There was a marked decrease in susceptibility of the flexed-tail animals over the normal-tailed animals bearing the dominant character from the susceptible strain A mice. This is evidence that the shaker-2 and flexed-tail genes are associated with chromosomes which in turn are associated with the susceptibility factor. Similar differences were noted when these crosses were compared as to the number of pulmonary nodules produced.

A similar experiment was made using waltzer and waved-1 genes. No significant evidence was found to associate these genes with susceptibility to induced pulmonary tumors.—R. C. R.

LAW, L. W. [Roscoe B. Jackson Memorial Lab., Bar Harbor, Maine] FOSTER NURSING AND THE GROWTH OF TRANSPLANTABLE LEUKEMIAS IN MICE. Cancer Research, 2:108-115. 1942.

The degree of resistance against progressive growth of inoculated malignant cells characteristic of certain refractory strains of mice has been reduced by foster nursing of the refractory mice by mothers of the susceptible strain. The influence present in the milk of Dba subline 2 mice has been shown to affect the growth of two transplantable lymphoid leukemias, line LL449 and line P1534. On the other hand, foster nursing was shown to be ineffective in influencing the growth of a myeloid leukemia, line LL493.

The "susceptibility" influence apparently is present in the milk of lactating females during the first 6 or 7 days of the lactation period. Data are not as yet available to determine the presence or effectiveness of the susceptibility influence during the latter part of the lactation period. Total deprivation of susceptible milk in young of the susceptible strain followed by foster nursing by females of the refractory strain does not affect the response of these mice to inoculations of leukemic cells.

A change in the growth capacity of the malignant cells of the two lymphoid leukemias, line LL449 and line P1534, and of the myeloid leukemia, line LL493, whereby leukemic cells can be grown by serial transfer in refractory mice has been observed. This occurs following initial growth of the malignant cells in refractory foster-nursed mice.—Author's summary.

LEWIS, M. R., and G. B. MIDER. [Carnegie Inst. of Washington, Baltimore, Md., Wistar Institute, Philadelphia, Pa., and Nat. Cancer Inst., Bethesda, Md.] SPONTANEOUS REGRESSION OF MYELOMAS AND OF THEIR METASTATIC GROWTHS. J. Nat. Cancer Inst., 2:123-125. 1941.

Three myeloid tumors produced by subcutaneously transplanting the buffy coat, particles of lymph nodes, or spleen of dilute brown mice with induced leukoses were found to be 100% transplantable in Bagg albino mice. In the dilute brown mice, as the tumor grew, a greenish fluid collected around the tumor and bilateral edema and ascites developed. The area of injection was characterized grossly by a flat, wide, hemorrhagic tumefaction. Metastases were frequent to lymph nodes and spleen. A similar picture except for absence of ascites was seen in the Bagg albino strain with transplants.

When the tumors remained in the dilute brown mice longer than 8 days the animal died. In the Bagg albino mice, however, the smaller tumors began to regress on the 8th day and had entirely disappeared in a few days thereafter. The larger tumors in this series of mice continued until the 10th day and then regressed. The

majority of these mice survived and proved to be immune to another transplant.

This indicates that the growth of a tumor graft is concerned with the genetic constitution of the host. It is suggested that perhaps biochemical differences in these two host mice may be shown to explain the difference in susceptibility.—R. C. R.

LOEB, L., and V. SUNTZEFF. [Washington Univ. Sch. of Med., St. Louis, Mo.] GROWTH PROCESSES IN MAMMARY GLANDS OF MICE OF STRAINS DIFFERING IN INCIDENCE OF MAMMARY CARCINOMA. Arch. Path., 32:739-759. 1941.

A statistical study of 756 mice, in which a microscopic examination of sex organs and certain other organs was made, confirmed the conclusion that the various strains of mice that reach the stage of cancerous growth of the mammary gland pass first from the phase of resistance to growth to a phase of preparatory growth and that the difference in the readiness with which the cancerous phase is reached depends not only on the readiness with which the mice pass from the stage of preparatory growth by way of a precancerous stage to the fully developed carcinoma but also on the readiness with which mice pass from the first phase, that of resistance to growth, to the preparatory growth phase and on the rapidity with which they pass through the stage of preparatory growth. The readiness with which the mammary glands of various strains respond with the progressive growth that is characteristic of the preparatory growth phase to long-continued stimulation by endogenous or exogenous estrogenic substances is at least one of the factors determining the difference in the hereditary tendency of various strains of mice toward the development of mammary gland carcinoma.—Authors' summary.

SLYE, M. [Sprague Memorial Inst. and Univ. of Chicago, Chicago, Ill.] HEREDITY AS DETERMINING THE TYPE AND SITE OF CANCER AND THE AGE AT WHICH IT OCCURS. Am. J. Path., 17:655-665. 1941.

The author presents evidence for the genetic influence in the occurrence of malignancy in mice with respect to the type of tumor, its site, and the age at which it will occur. The data are based on a study of 4 strains of mice and their hybrid crosses.

When mice of a strain with high incidence of malignant disease (whether carcinoma, sarcoma, or leukemia) were crossed with a strain with low incidence, the frequency of malignancy in the hybrids was observed to fall between the extremes. Crossing a sarcoma strain with one in which carcinoma occurred resulted in hybrids that demonstrated a medium incidence of both carcinoma and sarcoma. No extrachromasomal factor was observed following reciprocal crosses between high and low mammary cancer strains, or in a study of lung cancer. Inasmuch as mammary cancer could be eliminated in a certain strain which still showed a high incidence of carcinoma, the author feels that this is evidence for the existence of separate factors for the occurrence of malignancy and for its site of origin.

In studying hybrids between short and long-lived strains, longevity was a dominant; it was also found that the occurrence of malignancy late in life was dominant over the tendency to have malignancy early.—H. B.

Radiation

KENNEY, J. M. [Memorial Hospital, New York, N. Y.] RADIOACTIVE PHOSPHORUS AS A THERAPEUTIC AGENT IN MALIGNANT NEOPLASTIC DISEASE. Cancer Research, 2:130-145. 1942.

This paper summarizes the results obtained by a group of workers at the Memorial Hospital who investigated the use of radioactive phosphorus (P^{32}) as a therapeutic agent in cancer. The first portion of the paper presents the physical characteristics of P^{32} and discusses the expression of its radiation in terms of "equivalent roentgens." A differential absorption ratio is described which makes it possible to determine how much radiation a given tissue will receive after a known dose of P^{32}.

The absorption of P^{32} by normal tissues, bone, muscle, lymph nodes, skin, and fat, and by various malignant neoplasms, carcinoma of the breast, osteogenic sarcoma, lymphosarcoma, and leukemia, was measured. The findings indicated, according to the author, that P^{32} was preferentially absorbed by the tissue of osteogenic sarcoma, lymphosarcoma, and leukemia and could, therefore, be a useful therapeutic agent in these diseases.

Six patients with chronic myelogenous leukemia were treated with P^{32} with good results; 8 patients with chronic lymphatic leukemia were treated with fair results; 10 patients with acute or subacute leukemia were treated with poor results. The methods of therapy used in the different cases are described and the case histories of the various patients are briefly summarized. Graphs of the effect of P^{32} on the blood count are presented.

Twelve patients with lymphosarcoma were treated with P^{32} with encouraging results. Eight patients with osteogenic sarcoma received P^{32} in therapeutic amounts but the effect was inconclusive.

Cytological studies were made of tissues secured by biopsy or at autopsy, from patients who had received P^{32}, but no change in cellular structure was noted which could be ascribed to the isotope.

It is the author's belief that radioactive phosphorus is a useful therapeutic agent in leukemia and lymphosarcoma, but that its value in other forms of cancer cannot be determined until more time has elapsed.—Author's abstract.

LAWRENCE, E. A., and F. DURAN-REYNALS. [Yale Univ. Sch. of Med., New Haven, Conn.] THE EFFECT OF COMBINING BACTERIAL TOXINS AND X-RAY IRRADIATION IN THE TREATMENT OF A TRANSPLANTABLE MOUSE CARCINOMA. Yale J. Biol. & Med., 14:177-181. 1941.

Irradiation of a theelin-induced mammary carcinoma in strain A mice was combined with injections of various bacterial toxins. The lives of these animals were not prolonged over those receiving irradiation alone, nor did the toxins alone increase the survival time of tumor-bearing mice in contrast with untreated controls.

The toxins were obtained from bacteria-free filtrates of cultures of colon, paratyphoid B, and typhoid bacilli. In some instances repeated intraperitoneal injections of the toxins were made, and in others testicular extracts were used in combination with the toxins, but without striking effect.—A. A. L.

RUSCH, H. P., B. E. KLINE, and C. A. BAUMANN. [Univ. of Wisconsin, Madison, Wis.] THE NONADDITIVE EFFECT

OF ULTRAVIOLET LIGHT AND OTHER CARCINOGENIC PROCEDURES. Cancer Research, 2:183-188. 1942.

Small doses of different carcinogenic agents, each given during separate periods, were administered to mice or rabbits in order to determine whether their carcinogenic activities were additive. The carcinogenic process initiated by the application of benzpyrene, methylcholanthrene, or 9,10-dimethyl-1,2-benzanthracene to the backs of mice could be completed by either of the other two hydrocarbons. Ultraviolet light did not increase the number of tumors produced in mice rendered precarcinogenic with methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene, and the hydrocarbons failed to increase the number of tumors in animals which were precarcinogenic with respect to irradiation tumors. Certain hormones, vital dyes, and other substances likewise failed to increase the number of tumors induced by ultraviolet irradiation. Ultraviolet irradiation and x-rays both failed to increase the carcinogenicity of the Shope papilloma virus.—Authors' abstract.

Biochemistry and Nutrition—Chemotherapy

ABELS, J. C., A. T. GORHAM, G. T. PACK, and C. P. RHOADS. [Memorial Hosp., New York, N. Y.] METABOLIC STUDIES IN PATIENTS WITH CANCER OF THE GASTRO-INTESTINAL TRACT. I. PLASMA VITAMIN A LEVELS IN PATIENTS WITH MALIGNANT NEOPLASTIC DISEASE, PARTICULARLY OF THE GASTRO-INTESTINAL TRACT. J. Clin. Investigation, 20:749-764. 1941.

By means of the Carr-Price reaction (development of blue color produced by the reaction of the vitamin and antimony trichloride) the levels of vitamin A in the plasma of patients with gastrointestinal cancer were found to be below the normal range in 86% of the patients examined.

The plasma vitamin A of 62 normal males was 170 ± 38 U.S.P. units per 100 cc. and the plasma carotene was 0.21 ± 0.13 mgm. per 100 cc. For 62 normal females these values were 149 ± 46 U.S.P. units per 100 cc. for plasma vitamin A and 0.18 ± 0.10 mgm. per 100 cc. for carotene. As long as the individuals in this study, both normal and those with cancer, remained untreated and on the same diet, their plasma levels of vitamin A never varied more than ± 10 U.S.P. units from day to day.

In contrast to the above were the levels in the plasma of 38 males with cancer of the gastrointestinal tract: vitamin A ranged from 32 to 180 U.S.P. units per 100 cc. and averaged 84 U.S.P. units, and carotene ranged from 0.04 to 0.50 mgm. per 100 cc. and averaged 0.14 mgm. Thirteen female patients with cancer of the gastrointestinal tract provided similar findings: vitamin A ranged from 50 to 148 U.S.P. units per 100 cc. and averaged 78.5 U.S.P. units, and carotene ranged from 0.5 to 0.27 mgm. per 100 cc. and averaged 0.135 mgm.

The explanation for the low plasma levels of vitamin A is thought to be an inadequate ingestion and absorption of the vitamin, or an hepatic dysfunction in connection with the storage of vitamin A or its formation from carotene. A dietary deficiency or malabsorption could not account for the low plasma vitamin A values.

Patients who have had successful resection of gastrointestinal cancer have a lower incidence of reduced plasma levels of vitamin A than do patients in whom gastrointestinal cancer is still present. The administration of yeast, lipocaic, or choline chloride, themselves free of carotenoids, raised the reduced plasma vitamin A levels of the patients with gastrointestinal cancer.

Patients with other malignant diseases (lymphomas, cancer of the head of the pancreas, and bone sarcoma) also showed a high incidence of low plasma vitamin A levels.—J. L. M.

BALL, H. A., H. F. SCHOTT, and L. T. SAMUELS. [San Diego County Gen. Hosp., San Diego, Calif., Univ. of Southern California Sch. of Med., Los Angeles, Calif., and Univ. of Minnesota Med. Sch., Minneapolis, Minn.] GLYCOGEN IN WALKER TUMOR 256. Cancer Research, 2:146-149. 1942.

Chemical assay of glycogen in 92 Walker 256 tumors in intact and hypophysectomized rats yields values that are inversely correlated with tumor size and rate of growth. Elimination of the effect of hypophysectomy did not alter the correlation between glycogen content and rate of growth. The viable periphery of the tumor and the tumor as a whole, including the necrotic center, yield glycogen values that are almost identical and which show a high degree of positive correlation. Chemical methods appear to demonstrate the presence of glycogen in amounts greater than would be inferred from histological preparation where particulate (undissolved) glycogen seems to be the only form shown.—Authors' abstract.

CARRUTHERS, C. [Barnard Free Skin and Cancer Hosp., St. Louis, Mo.] THE EFFECT OF CARCINOGENS ON THE HEPATIC VITAMIN A STORES OF MICE AND RATS. Cancer Research, 2:168-174. 1942.

The influence of repeated biweekly intraperitoneal injections of methylcholanthrene and phenanthrene for 18 weeks was without effect upon the hepatic vitamin A stores in mice, although tumors and ascites were induced by methylcholanthrene. Large doses of phenanthrene, 3,4-benzpyrene, methylcholanthrene, and 1,2,5,6-dibenzanthracene were also without effect upon the hepatic vitamin A stores when the latter were maintained at a high level by the oral administration of vitamin A to mice.

The intraperitoneal administration of large doses of 3,4-benzpyrene and methylcholanthrene to rats induced a marked lowering of the hepatic vitamin A content which was much greater than the inhibition of growth. The inhibition of growth by phenanthrene somewhat paralleled the slight decrease in the hepatic vitamin A content. —Author's abstract.

DICKENS, F. [Cancer Research Lab., North of England Council of the British Empire Cancer Campaign, Royal Victoria Infirmary, Newcastle-upon-Tyne] THE CITRIC ACID CONTENT OF ANIMAL TISSUES, WITH REFERENCE TO ITS OCCURRENCE IN BONE AND TUMOUR. Biochem. J., 35:1011-1023. 1941.

The citric acid content of numerous tissues was found to be as follows (mgm./100 gm.): Rabbit tissues: Brown-Pearce carcinoma 13.6; (? same rabbit) liver 2.8, skeletal muscle 2.5, kidney 6.0, whole brain 4.6. Rat tissues: Walker No. 256 carcinoma 16.6, 18.4; Guérin tumor 16.8, liver (same animal) 3.0. Mouse tissues: Crocker No. 180 sarcoma 14.2, 14.3; mainly nonnecrotic part of a Crocker tumor 12.3, mainly necrotic part (same animal) 7.7; normal tissues (probably different animals from tumor-bearers) skin 12.2, fur 133, seminal vesicles 128, skeleton 46. Guinea pig tissues: liver 1.6, kidney 3.9, whole brain

3.8, testis 11.5. Human tissues: seminoma of testis 9.9; uterine fibroadenoma 5.0; stomach carcinoma 2.7 (adjacent gastric mucosa 2.0); carcinoma of vulva 8.8, 7.0; leukoplakia of vulva 8.2 (adjacent normal skin 5.3).

In whole chick embryo the citric acid falls from 31 at 3½ days to 9.4 at 21 days.

Thus of all tissues studied, fur (maximum 133), bone (after drying and alcohol extraction, maximum 1660), and seminal vesicles (maximum 633) are much the richest in citric acid.

The original paper should be consulted for a very full account of the technic of assay and for a discussion of the role of citric acid in calcium metabolism.—I. H.

DOBRINER, K., G. I. LAVIN, and C. P. RHOADS. [Memorial Hosp., and Hosp. of the Rockefeller Inst. for Med. Research, New York, N. Y.] THE SPECTROSCOPIC STUDY OF BIOLOGICAL EXTRACTS. I. URINE. Cancer Research, 2:79-94. 1942.

A method for the treatment and preliminary separation of the ether-soluble substances normally present in urine into groups suitable for the study of absorption spectra is described. Following such a separation, absorption spectra do not present the confused picture of overlapping bands which has been found in the past with whole urine. While no claim is made for complete identification of any one substance, its probable nature may be inferred from comparison with the absorption spectra of certain aromatic substances which are known from previous chemical study to be present in normal urine.

If these pure aromatic substances are mixed and subjected to the fractionation procedures, the resulting absorption bands in the different fractions are in similar positions to the absorption bands from the same fraction obtained from the urine.

Thus far, we have had occasion to study some 150 different urines from 48 normal and diseased individuals, and have not encountered appreciable variations from the findings presented. Possibly the same sort of separation and classification will enable a clearer picture of the absorption spectra of other body fluids to be made.—Authors' summary.

DOBRINER, K., C. P. RHOADS, and G. I. LAVIN. [Memorial Hosp. and Hosp. of the Rockefeller Inst. for Med. Research, New York, N. Y.] THE SPECTROSCOPIC STUDY OF BIOLOGICAL EXTRACTS. II. THE DETECTION, ISOLATION, AND BIOLOGICAL EFFECTS OF THE METABOLITES OF 1,2,5,6-DIBENZANTHRACENE. Cancer Research, 2:95-107. 1942.

The fate of dibenzanthracene was investigated in mice, rats, rabbits, dogs, and monkeys by chemical and spectroscopic methods. In the excreta of mice, rats, and rabbits, evidence was obtained spectroscopically for the presence of phenolic derivatives of dibenzanthracene. In all species investigated, no suggestion of a conjugated derivative of dibenzanthracene has been found. The presence of dibenzanthracene and derivatives was investigated spectroscopically in tissues.

The phenolic derivatives of dibenzanthracene excreted by mice, rats, and rabbits were isolated and the physical and chemical properties showed that the compounds excreted by mice and rats are identical. Evidence is presented for the identity of the dibenzanthracene metabolite excreted by mice and rats with 4′,8′-dihydroxydibenzanthracene.

Rabbits excrete a dihydroxydibenzanthracene which is not identical with that excreted by mice and rats. The derivative excreted by rabbits is without carcinogenic activity when injected into mice. The biological significance of the conversion of dibenzanthracene to phenolic metabolites is discussed. The usefulness of a combination of spectroscopic and chemical methods is pointed out.—Authors' abstract.

GOTH, A. [Vanderbilt Univ. Sch. of Med., Nashville, Tenn.] CONJUGATION OF SULFANILAMIDE BY A PATHOLOGICAL TISSUE IN VITRO. J. Pharmacol. & Exper. Therap., 74: 71-74. 1942.

Fowl-pox lesion or epithelioma, liver, and, in some experiments, skin were removed from chicks from 7 to 16 days after inoculation with fowl-pox virus. Thin sections of each tissue were placed in Ringer-phosphate, Ringer-bicarbonate, or Ringer-acetate solution to which dextrose and sulfanilamide were then added in 0.2% and 0.02% concentration respectively. After shaking for 5 hours at 39° C. in the Warburg apparatus the fluid in each flask was analyzed for free and total sulfanilamide. The conjugating capacity (mgm. of sulfanilamide conjugated per 1 mgm. dry tissue) of the fowl-pox lesion was significantly greater than that for normal skin and of the same magnitude as that for liver. Since the fowl-pox lesion has been reported to possess the ability to split glucose both anaerobically and aerobically and lactic acid is the probable precursor of the acetic acid shown to be necessary for conjugation of sulfanilamide, such conjugating capacity should be considered in connection with malignant tumors.—F. L. H.

GREEN, E. U. [Bryn Mawr Coll., Bryn Mawr, Pa.] ON THE OCCURRENCE OF CRYSTALLINE MATERIAL IN THE LUNGS OF NORMAL AND CANCEROUS SWISS MICE. Cancer Research, 2:210-217. 1942.

A study has been made of the nature and incidence of crystalline material found within or associated with the phagocytic cells of the lungs of mice of the Swiss strain. The crystal substance proved to be a protein.

A few crystals are found in stock mice 3 weeks old. The number increases with age, giving a correlation coefficient, r_{ca}, of 0.64. There is a still closer correlation between the quantity of crystals and the volume of spontaneous pulmonary tumor ($r_{ct}=0.76$). In mice injected subcutaneously with 1,2,5,6-dibenzanthracene, the incidence of both crystals and tumors is increased. However, the rise in crystals does not occur until several months after the development of multiple tumors.

Crystals were found in the lungs of mice of strains other than the Swiss. The incidence in these strains does not show a positive correlation with the incidence of lung tumors. Mice of the Bagg albino and A strains, both relatively susceptible to lung tumors, show crystals in a rather small percentage of individuals. There is some evidence that the quantity of crystals is increased in Bagg mice by injection with 1,2,5,6-dibenzanthracene.

Attempts to influence the occurrence of crystals by intranasal inoculation of extracts of lung tissue have so far been without effect, and the tumor incidence in inocu-

lated and uninoculated mice was approximately the same. Further experiments along this line are in progress.

The occurrence of consolidation of portions of one or more lobes of the lung in inoculated mice suggests the presence of an agent which might account for the condition of chronic inflammation with which the crystals are associated. The agent did not become lethal on repeated passage.

The possible relations of the crystals to inflammation and tumors are discussed. From the evidence as it stands no positive correlation between any of these factors can be assumed.—Author's abstract.

GUTMAN, A. B., D. H. MOORE, E. B. GUTMAN, V. McCLELLAN, and E. A. KABAT. [College of Physicians and Surgeons, Columbia Univ., New York, N. Y.] FRACTIONATION OF SERUM PROTEINS IN HYPERPROTEINEMIA, WITH SPECIAL REFERENCE TO MULTIPLE MYELOMA. J. Clin. Investigation, 20:765-783. 1941.

Analyses of serum proteins by means of the salting-out technic (Howe method) and electrophoresis (Tiselius method) were carried out in normal adults, in patients with hyperproteinemia due to various chronic infections or to cirrhosis, and in patients with multiple myeloma.

The following results were obtained when the serum proteins of 38 cases of multiple myeloma were partitioned by the Howe method. In some cases hyperglobulinemia resulted from an increase in both euglobulin and pseudoglobulin I fractions. These increases were of the same type found in the hyperproteinemia occurring in patients with lymphogranuloma venereum, cirrhosis, sarcoid, and miscellaneous infections. In other cases anomalous results were obtained: the increase involved only the pseudoglobulin I, without any accompanying rise in euglobulin; or the pseudoglobulin II or albumin fractions exceeded normal limits. Electrophoretic analyses in 10 cases of multiple myeloma also gave varied results: an increase in components moving with the mobility of gamma-globulin, of beta-globulin, or of a globulin with an intermediate mobility. These anomalous Howe and electrophoretic patterns were found to be of value in diagnosis.

The significance of these peculiarities is brought out in a study of the analogous salting-out and electrophoretic properties of urinary Bence-Jones proteins obtained from patients with multiple myeloma. The variations found correspond with the anomalies observed in the salting-out and electrophoretic behavior of myelomatous serums. The addition of urinary Bence-Jones proteins from different patients to normal serum produced the essential characteristics of some anomalous Howe patterns and of all electrophoretic patterns observed in myelomatous serums.

Myelomatous serums fall into 3 groups: (1) Serums with hyperglobulinemia due to an increase in the euglobulin and pseudoglobulin I fractions; electrophoretically, to an increase in the gamma-globulin. The globulin increment in these cases include little or no Bence-Jones protein, and is comparable to that observed in chronic infections. (2) Serums with or without hyperglobulinemia giving a variety of patterns by the salting-out or electrophoretic methods, for the most part due to Bence-Jones proteinemia. (3) Serums of apparently normal composition with respect to serum proteins.—J. L. M.

JACOBI, H. P., and C. A. BAUMANN. [Univ. of Wisconsin, Madison, Wis.] CHOLINE IN TUMOR-BEARING ANIMALS AND A CHOLINE-LIKE EFFECT OF BUTTER YELLOW. Cancer Research, 2:175-180. 1942.

Analyses for free choline, total choline, and organic P revealed that less than half of the tumor phospholipids were present as lecithin. The tissues of tumor-bearing animals contained normal amounts of both free and bound choline. A choline deficiency failed to alter the course of development of the two types of tumors studied, and an excess of choline was equally without effect.

The addition of butter yellow to a choline-free ration prevented the development of kidney lesions in young rats. The effect was not due to a reduced food intake, nor could it be duplicated with aminoazotoluene. Thus, like certain other compounds containing labile methyl groups, butter yellow may act as a "methyl donor."—Authors' abstract.

MACLEAN, I. S., and L. C. A. NUNN. [Lister Inst., London, England] FAT-DEFICIENCY DISEASE OF RATS. THE RELATION OF THE ESSENTIAL UNSATURATED ACIDS TO TUMOUR FORMATION IN THE ALBINO RAT ON NORMAL DIET. Biochem. J., 35:983-989. 1941.

Rats fed from the time of weaning on a diet free from fat cease to gain in weight and develop a scurfy skin and the condition of "scaly tail." They can be cured by small doses of linoleic or arachidonic acids, the latter being the more potent. The bodies of such rats, although in a fat-starved condition, show an abnormally high amount of fat. Growth is resumed, and the abnormal amount of fat disappears, after methyl arachidonate (7 to 14 mgm. daily) is given. Hence these unsaturated acids appear to be concerned in the formation of new tissue.

Estimations were made of the total lipoid and polyunsaturated acids (reckoned as arachidonic acid) in the skin, liver, and remaining carcass of rats receiving a diet rich in fat and unsaturated acids. The average unsaturated acid of the skin, reckoned as arachidonic acid and as a percentage of the fat-free dry weight of tissue was, in 22 control rats, 1.33; in 14 rats bearing the Walker tumor, 0.82.

In another experiment all the Walker tumors implanted failed to grow, or regressed; these rats were not examined but 3 of the controls showed the very low average figure of 0.31 for the same percentage. No such differences were detected in the fats of the liver and carcass.—E. L. K.

MACLEAN, I. S., and E. M. HUME. [Lister Inst., London, England] FAT-DEFICIENCY DISEASE OF RATS. THE STORAGE OF FAT IN THE FAT-STARVED RAT. Biochem. J., 35:990-995. 1941.

Data are given on the storage of fat, and of unsaturated acids in the skin, liver, and carcass of fat-starved rats, and on the effects of administration of methyl arachidonate.—E. L. K.

MACLEAN, I. S., and E. M. HUME. [Lister Inst., London, England] FAT-DEFICIENCY DISEASE OF RATS. THE INFLUENCE OF TUMOUR GROWTH ON THE STORAGE OF FAT AND OF POLYUNSATURATED ACIDS IN THE FAT-STARVED RAT. Biochem. J., 35:996-1002. 1941.

Walker tumors were implanted (a) in 7 rats which had been maintained for 97 days on a fat-free diet, and (b) in 7 rats which had received for the same period daily doses of linseed oil in addition to the fat-free diet. Large tumors developed in all the fat-starved rats and in 5 of

those which had received the linseed oil. In 2 rats receiving linseed oil, small nodules formed but failed to develop further. The 5 "linseed oil" rats in which tumors grew well showed a lower average proportion of lipoid (skin 44.6%, carcass 23.4% of fat-free dry weight) than that in 2 rats in which only small nodules developed (skin 90.4%, carcass 34.5%). In the fat-starved rats, the percentage of lipoid was lower in those bearing tumors (skin 28.6 to 42.2%, carcass 20.3 to 26.7%) than in those in which no implantation had been made (skin 65.8 to 98.3%, carcass 30.8 to 46.4%). A considerable lowering of highly unsaturated acid in the skin, but not in the carcass, accompanied the development of tumors.—E. L. K.

WILLIAMSON, R. [Dept. of Pathology, Cambridge, England] A NOTE ON THE EPIDERMIS OF THE RAT ON A FAT-FREE DIET. Biochem. J., 35:1003-1005. 1941.

The epidermis of rats on fat-free diets is thickened and the stratum granulosum is very distinct; if such a diet were supplemented with unsaturated fatty acids the epidermis was more nearly, or quite, normal.—E. L. K.

TAYLOR, A., and M. A. POLLACK. [Biochem. Inst., Univ. of Texas, Austin, Texas] HEMOGLOBIN LEVEL AND TUMOR GROWTH. Cancer Research, 2:223-227. 1942.

A study was made of the effect of the following conditions on the blood hemoglobin level: 1. growth of tumor transplants in mice, 2. methylcholanthrene deposits in mice, 3. ingestion of butter yellow by rats in conjunction with diets of Purina dog chow and cooked whole rice and raw carrots.

The results obtained led to the conclusion that tumor tissue has an antagonistic action on the hemoglobin of the host. The hemoglobin level was depressed by the time the implant had grown to measurable size and thereafter became progressively lower until death intervened.

The precancerous condition as induced by methylcholanthrene was associated with a gradual fall in blood hemoglobin concentration in both cancer-susceptible and cancer-resistant mice indicating that precancerous tissue was also antagonistic to hemoglobin in the degree to which it approached the cancerous state.

Ingestion of butter yellow by rats induced a sudden drop in the hemoglobin to a new level in the first few weeks and thereafter it remained constant over a total period of 162 days until hepatoma developed. The hemoglobin of butter yellow-fed rats on a rice-carrot diet dropped to a much lower level than was true for butter yellow-fed rats on a diet of Purina dog chow.—Authors' abstract.

TENNANT, R., K. G. STERN, and A. A. LIEBOW. [Yale Univ. Sch. of Med., New Haven, Conn., and Meriden Hosp., Meriden, Con.] THE STIMULATING ACTION OF NUCLEIC ACIDS UPON MOUSE HEART FIBROBLASTS. Cancer Research, 2:218-222. 1942.

Sodium nucleinates from various sources significantly increase the expansion rates of mouse heart fibroblasts at concentrations in the medium of about 1:50,000. This is evidence that the nucleinates represent one stimulating factor of embryo extract or its derivative macromolecular material. The thermal or enzymic breakdown of the macromolecular material is calculated to yield at least the effective concentration of nucleinates.

These observations do not intimate that other components of embryonic extract may not also have a stimulating action.—Authors' summary.

STEINER, P. E. [Univ. of Chicago, Chicago, Ill.] THE NON-SAPONIFIABLE LIPID FRACTION OF LIVERS FROM CANCEROUS AND NON-CANCEROUS PERSONS. Am. J. Path., 17:667-671. 1941.

The author previously described the production of spindle cell sarcomas in mice from the subcutaneous injection of the nonsaponifiable lipid fraction of livers from persons with cancer. This paper presents in detail the method of making such extracts. Although individual variation is great, no significant differences have been found in the per cents of nonsaponifiable lipids as obtained from 33 livers of cancerous persons in contrast to 19 noncancerous.—H. B.

STEVENSON, E. S., K. DOBRINER, and C. P. RHOADS. [Memorial Hosp., New York, N. Y.] THE METABOLISM OF DIMETHYLAMINOAZOBENZENE (BUTTER YELLOW) IN RATS. Cancer Research, 2:160-167. 1942.

The metabolism of N,N′-dimethylaminoazobenzene (butter yellow) was investigated in rats. The following compounds have been isolated and identified: *p*-aminophenol, N-acetyl-*p*-aminophenol, *p*-phenylenediamine, and N,N′-diacetyl-*p*-phenylenediamine. An additional metabolite, as yet unidentified, has been isolated. The significance of the splitting of the azo linkage and the demethylation of N,N′-dimethylaminoazobenzene in relation to the carcinogenic activity of this compound and its metabolites is discussed.—Authors' summary.

Clinical and Pathological Reports

Urinary System—Male and Female

GRAVES, R. C., and L. W. GUISS. [Pondville State Hosp., Walpole, Mass.] TUMORS OF THE URETHRA. J. Urol., 46:925-947. 1941.

At the Pondville Hospital, which is devoted to the care of malignant disease, there were but 1 case of carcinoma of the male urethra and 10 cases in the female in almost 19,000 admissions. The treatment of these cases is discussed in detail, and the conclusion drawn that radium treatment is better than surgery in early cases. Metastases to the regional nodes occur relatively late.—H. G. W.

KIRWIN, T. J. [New York Hosp., New York, N. Y.] PAPILLARY CARCINOMA OF THE RENAL PELVIS. Surg., Gynec. & Obst., 73:759-765. 1941.

Neoplastic growth in the renal pelvis extends rather to the ureter than to the renal cortex, so complete nephroureterectomy is the procedure of choice. Case report.—H. G. W.

LADD, W. E., and R. R. WHITE. [Harvard Med. Sch., Boston, Mass.] EMBRYOMA OF THE KIDNEY (WILM'S TUMOR). J. A. M. A., 117:1858-1863. 1941.

A study based on 60 cases, the oldest patient being but 9 years, the youngest 2 months. This is a marked contrast to hypernephroma, for in this hospital no case has been

seen in a child under 12 years old. The distribution between the two sexes and the two sides is nearly equal. Of the 60 cases, 18 are still living, 14 having probably been cured, and 4 operated on too recently to be certain. The size of the tumor bears little relation to the prognosis, but gross hematuria, or involvement of the renal vein, are unfavorable. Of 32 recurrences, 31 were prior to the 9th month after operation. If operation is performed as soon as the diagnosis is made, the chance of cure is greater than if it is delayed until after roentgen therapy has been given. Insufficient data are available for evaluation of the merits of postoperative roentgen therapy.—H. G. W.

Oral Cavity and Upper Respiratory Tract

CAULK, R. M. [Garfield Memorial Hosp. and Warwick Memorial Clinic, Washington, D. C.] TOMOGRAPHY OF THE LARYNX. Am. J. Roentgenol., 46:1-10. 1941.

The value of tomographic x-ray studies of the larynx is pointed out as an aid in determining the site of origin and extent of laryngeal tumors and diagnosing other forms of laryngeal disease.—C. E. D.

CID, J. M., and J. L. BONILLA, JR. [Rosario Med. Sch., Rosario, Argentina] GIANT CELL TUMOR OF THE TRACHEA. J. Thoracic Surg., 11:210-215. 1941.

Report of a unique case of giant cell sarcoma originating from an area of metaplastic ossification in the trachea of a man of 25. No record of a similar case could be found in the literature.—H. G. W.

CROMARTIE, W. J., and O. G. NELSON. [Vanderbilt Univ. Sch. of Med., and Protestant Hosp., Nashville, Tenn.] ADENOCARCINOMA OF THE TONGUE ARISING FROM VESTIGE OF MEDIAN ANLAGE OF THE THYROID GLAND. Arch. Surg., 43:599-608. 1941.

In this case, the vestige of tissue had remained embryonic in structure. It invaded the muscles of the tongue and metastases were found in the lungs. There is a brief review of other similar cases.—G. De B.

LENZ, M. [New York, N. Y.] CAUSES OF FAILURE OF ROENTGEN THERAPY IN CANCER OF THE LARYNX. Am. J. Roentgenol., 46:21-29. 1941.

Follow-up studies of 166 cases of laryngeal cancer treated by radiation at the Presbyterian Hospital and Montefiore Hospital between 1926 and 1938 showed that 118 were dead in 1940, 13 were alive with disease, and 35 were alive and free of disease. Eleven tables are presented showing the location, extent, histological grade, presence of metastases, etc. of the various tumors in relation to their outcome, in an attempt to determine the reasons for therapeutic failures. In general, failure was attributable to local invasion of the tumor, particularly into the arytenoid cartilages or base of the tongue, massive local extension, regional lymph node involvement, poor general condition of the patient, or technical errors in treatment.—C. E. D.

MARTIN, H. [Memorial Hosp., New York, N. Y.] CANCER OF THE GUMS (GINGIVAE). Am. J. Surg., (Special Monograph), 54:770-806. 1941.

A monographic discussion of 113 cases of cancer of the gums, from the standpoint of etiology, clinical course, treatment, and end results. Treatment methods by radiation, surgery, and combinations of the two, with their attendant complications, are described in detail. The net cure rate in this series is 25.7%.—H. G. W.

MARTIN, H., and C. L. GRAVES. [Memorial Hosp., New York, N. Y.] PLEXIFORM NEUROFIBROMA (VON RECKLINGHAUSEN'S DISEASE) INVADING THE ORAL CAVITY. J. A. M. A., 117:1535-1539. 1941.

In 2 cases of generalized neurofibromatosis there was bulky invasion of the oral cavity, in one case malignant without metastases, the other apparently benign. Both cases were in children.—H. G. W.

QUICK, D. [New York, N. Y.] TREATMENT OF LARYNGEAL CANCER BY IRRADIATION. Am. J. Roentgenol., 46:11-20. 1941.

Adequate roentgen therapy offers an excellent chance for cure in laryngeal cancer and is applicable to a larger group of cases than is radical surgery. An added advantage is preservation of laryngeal function. The histological grade of the tumor influences therapy but little, since the dose of radiation should approach the limit of normal tissue tolerance. The technic of roentgen irradiation and the indications for radon seed implantation are discussed. Metastases to cervical lymph nodes are infrequent and may be treated by surgical exposure and radon implantation.—C. E. D.

SCHALL, L. A. [Boston, Mass.] CANCER OF THE LARYNX; SURGICAL TREATMENT. Am. J. Roentgenol., 46:30-32. 1941.

Surgical treatment of cancer of the larynx should be limited to cases with intrinsic lesions of a low histological grade of malignancy and without regional adenopathy.

Small localized lesions in the anterior portion of the larynx are amenable to laryngofissure and local removal while extensive intrinsic lesions receive total laryngectomy. When patients are selected according to these criteria, surgery offers better than 75% 3- to 5-year cures.—C. E. D.

Intrathoracic Tumors—Lungs—Pleura

MENNE, F. R., and M. W. ANDERSON. [Univ. of Oregon Med. Sch., Portland, Oreg.] BRONCHIOGENIC CARCINOMA. J. A. M. A., 117:2215-2221. 1941.

A study of the incidence of this tumor in the Pacific northwest leads to the conclusion that there has been an absolute increase in recent years. Of 84 instances of bronchiogenic carcinoma observed in the authors' laboratory, 78 were in men and but 6 in women, and the youngest patient was 38 years old. In 56 cases with necropsy, 31 were squamous cell carcinoma, 13 adenocarcinoma, and 12 reserve cell carcinoma. Macroscopically they may be classified into two types, hilar nodular and diffuse necrotic. It would seem that the causative agent is air-born and in a volatile state, and emphasis is laid on the etiologic significance of inflammatory processes augmented by tobacco smoke and the gaseous products of the industries.—H. G. W.

RAINE, F. [Marquette Univ., Milwaukee, Wis.] METASTATIC CARCINOMA OF THE LUNG INVADING AND OBSTRUCTING A BRONCHUS. J. Thoracic Surg., 11:216-218. 1941.

Report of a case in which a metastasis from a carcinoma of the sigmoid had the usual location and effects of a primary bronchiogenic carcinoma, and was removed by

pneumonectomy. Only two other cases of similar metastatic tumors, both hypernephromas, could be found in the literature.—H. G. W.

WOMACK, N. A., and E. A. GRAHAM. [Washington Univ. Sch. of Med. and Barnes Hosp., St. Louis, Mo.] EPITHELIAL METAPLASIA IN THE CONGENITAL CYSTIC DISEASE OF THE LUNG. ITS POSSIBLE RELATION TO CARCINOMA OF THE BRONCHUS. Am. J. Path., 17:645-653. 1941.

The authors describe 3 specimens of congenital cystic disease of the lung in which foci of epithelial metaplasia were present within the bronchial walls. These had apparently arisen from the epithelium of the air sacs and had grown into the bronchial walls. Such foci were composed of cells of the squamous type, were nonencapsulated but showed little or no evidence of mitotic activity; no metastases were demonstrable in the hilar lymph nodes or elsewhere in the body.

The authors state that these findings represent a "disturbance in the fundamental structural tissue growth" as often seen in areas of abnormal tissue organization. The relation between such abnormal epithelial proliferation and cancer will be discussed by them at a later date.—H. B.

Gastrointestinal Tract

BARGEN, J. A., C. D. L. CROMAR, and C. F. DIXON. [The Mayo Clinic, Rochester, Minn.] EARLY CARCINOMA OF THE COLON (Part 1). Arch. Surg., 43:186-191. 1941.

This subject is discussed in two papers. The first paper is a study of 61 colonic cancers of less than 2.5 cm. in diameter. Various operative procedures were used which were as radical as those usually employed for larger lesions. Of the 61 patients, 28 were alive 5 years after operation, but only 24 were free of carcinoma.

Because carcinoma recurred in some cases in which adequate resection was thought to have been done, the authors studied the microscopic sections of these recurrent tumors. They conclude that in many cases an entirely new carcinoma forms. This part of the study appears in the second paper.—G. De B.

BARGEN, J. A., C. D. L. CROMAR, and C. F. DIXON. [The Mayo Clinic, Rochester, Minn.] EARLY CARCINOMA OF THE COLON (Part 2), Arch. Surg., 43:192-208. 1941.

In this paper the authors examine the relationship between subclinical inflammation and carcinoma. They studied 30 excised surgical specimens which on microscopic observation showed that the cancer had been entirely removed. Of these patients, 16 were alive and well 5 years after operation, 5 had died of metastases, and 9 had local recurrence.

In the areas surrounding colonic cancers, the authors found enlargement of the lymphoid follicles with damage to the muscularis mucosae. Later large encapsulated follicles with secondary centers developed, and these produced changes in the epithelial cells. When the follicles ruptured, some of the epithelial cells were displaced into the submucosa and enveloped by the lymphoid follicles. These mucosal cells were then pinched up into the form of polyps which are inflammatory and not true adenomas.

The authors conclude that a large "potentially malignant" area exists in the colon at the time when cancer develops. This area represents a reaction of the tissues to some extraneous influence. Since the reaction is inflammatory, the mesoblastic cells are primarily concerned, because they are the natural defensive cells of the body. The effect on the epithelial cells is secondary and represents a stage of impaired nutrition or of partial destruction, followed by a stage of attempted repair. This process of repair may become deranged if a part of the epithelium is isolated by a ring of lymphoid tissue, as occurs in the formation of polyps, or if epithelial cells become displaced into the submucosal tissue. This repair process in cells removed from their normal location may give rise to malignant anaplasia. Thus, malignant change in epithelial cells is not the result of chronic irritation but of change of environment of cells.—G. De B.

BOYCE, F. F. [Charity Hosp., New Orleans, La.] CARCINOMA OF THE STOMACH IN A LARGE GENERAL HOSPITAL. J. A. M. A., 117:1670-1675. 1941.

An analysis of the results in 1921 cases of carcinoma of the stomach treated in the Charity Hospital of Louisiana at New Orleans. Over a period of years only 2 of every 30 patients could be submitted to gastrectomy, and the immediate operative mortality was over 50 per cent. The 200 most recent surgical patients present very little improvement over a similar series reported 8 years ago. The most practical single method of reducing mortality in cancer of the stomach is to shorten the interval between the time the patient first consults the physician and the time the operation is performed.—H. G. W.

CRESSY, N. L. [Meriden Hosp., Meriden, Conn.] CARCINOMA OF THE DUODENUM. Connecticut M. J., 5:818-820. 1941.

This is a report of a patient with carcinoma of the intestinal mucosa covering the papilla of Vater, with pulmonary metastases.—G. De B.

CROSSMAN, L. W., and J. H. KIDDER. [New York City Hosp., New York, N. Y.] ADENOMYOMA OF THE STOMACH. Am. J. Surg., 54:395-402. 1941.

Report of a case of adenomyoma of the stomach containing glands of the Brunner type and producing a tumor of the pyloric antrum which eventually led to obstruction and 24-hour retention. The condition is rare, only one other case being found in the American literature.—H. G. W.

GARIS, R. W. [Baltimore, Md.] PRERENAL UREMIA DUE TO PAPILLOMA OF THE RECTUM. Ann. Int. Med., 15:916-926. 1941.

A case is reported of a large, essentially benign, nonobstructing tumor of the rectum which caused prerenal azotemia, presumably from excessive secretion by the neoplasm with progressive electrolyte loss and dehydration.—H. G. W.

GOLDEN, T., and A. P. STOUT. [Presbyterian Hosp., New York, N. Y.] SMOOTH MUSCLE TUMORS OF THE GASTROINTESTINAL TRACT AND RETROPERITONEAL TISSUES. Surg., Gynec. & Obst., 73:784-810. 1941.

A study based on 60 cases, of which 33 were in the stomach. Thirty caused clinical symptoms and all but 8 were treated by surgical removal; 21 of these were malignant and 9 benign. Classification of these tumors into benign and malignant is difficult, and the terms leiomyoma and malignant leiomyoma are preferred to any others proposed.—H. G. W.